Reproduction and Establishment of Two Endangered African Cedars, *Widdringtonia cedarbergensis* and *Widdringtonia whytei.*

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Thesis presented in fulfilment of the Master of Sciences degree in the Department of Biological Sciences University of Cape Town

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University of Cape Town

Contents

Acknowledgments

Many many people have been involved in helping me through the scientific and emotional side of this thesis. In submitting my thesis for examination, I not only enter the community of knowledge, but thank my community who supported me along the way and whom I would not have managed without.

In no specific order, thank you to;

Nicolas Zalumous and **Nikki Stevens** for having me run into their office every few minutes for a couple of days to ask about TZ staining. **Benny Lemaire** for teaching me labwork and helping me run samples when my anxiety got too much. **Tracey Nowell** for so much lab support and always being there to help and answer my questions. I couldn't have done it without her. **Vincent Naude**, who was in the lab when I was going crazy and helped me run samples when I was crippled by fear. **Lisa Nupen** who was super helpful and friendly in teaching me to use PeakScanner. **Shaamielah Davids** for all the technical help and moral support. **Nazlie Naude** for technical support. **Simon Power** for encouragement, support and help with statistics. **Kobus Kellerman** for all the support and comradery. **Matthew Trisos**, for statistical support and comradery. **Annabelle Rogers**, for helping out with fieldwork (some of it quite hair-raising) and for comradery. **Eleanor Shadwell**, a fellow soldier in the trenches, always a hug and an ear even when you were suffering under your own workload. Thank you for all the encouragement. **Rosemary Newton** who gladly gave of her extensive knowledge of seed germination and viability and took time to answer several rather long and detailed emails. **Tembo Chanyega** for the correspondence and field support in Malawi and to everyone at the Forestry department **and Mulanje Mountain Conservation Trust** for their help, knowledge and guidance. **Russell Croft** and **Nicolas Cooper** for climbing rather big trees for me, and providing comradeship on a grueling but awesome fieldwork trip and for **Kew Gardens** and **Millenium Seed Bank** for letting me piggyback on their excursion and skill. **PERC** for financially providing for this opportunity and the **NRF** for valuable financial support. **Heath Beckett**, who patiently and even enthusiastically, walked me through creating maps on GIS and who let me work in his office when they were drilling above mine. Sorry for all the distractions. **Sandy-Lynn Steenhuisen**, for lots of moral support and for the timely statistical support. **Jess McCarthy**, who always knew I could. (also for editing chapters). **Ruth Browne**, for all the trail runs that kept me sane, for listening to me when I cried, and who didn't care about my results. (also for editing chapters).

Susan Mitrani, fed me, loved me and got up at 4 in the morning when my inner fears and demons started having a tea party. I couldn't have done it without you, Mom.

Uri Mitrani, My father, who said “finish it first. For you. You can throw the degree in the bin afterwards and walk away, but don’t quit” and who also was awoken on many early morning, and knows best this is when the inner demons like to dance and the fears demand feeding.
And to both **Sue** and **Uri** for the rather massive financial support in terms of fees, food and a home, and medical care. It was a massive load off my shoulders
Talia Runciman, my sister, who walked the dogs when I had too much work, who loved me all the way through and loves me still.
Toby Runciman, my brother in law, for having been there for me and before me.
Edmund February. The four years of my thesis correlate strangely with the rapid greying of his hair. He really puts a vast amount of effort into all his students. He is a phenomenal supervisor. I couldn’t have done it without you, Ed. I am so grateful, more than you know.
Terry Hedderson, who in terms of workload, knowledge and energy expended should technically have been made a co-supervisor. I really appreciate all the input despite the official lack of credit. Who also had a smile, and was always relaxed no matter how much my anxiety drove me to his office with a hundred little questions.
Patrick Lane, of Cape Nature (Cedarberg Wilderness Area), who unfortunately never saw the end of my thesis as he died looking after the mountains he loved. He was always helpful and supportive, friendly and enthusiastic and greatly helped facilitate my project. He used to say in reference to the potential extinction of the Clanwilliam cedar “Not on my watch.” Unfortunately, his watch is ended now. It a great loss for the Cedarberg.
To all the **staff of the Cedarberg Wilderness Area** and **Cape Nature**. Especially the rangers who came with me on fieldwork, showed me where the trees were and tolerated my atrocious Afrikaans, I’m sorry if you thought I was murdering your language more than saving your trees. To my psychologist, **David Van Der Want** who is so good at his job I would like to clone him and dispense him all over the world. Thank you also to my psychiatrist for prescribing the right medication and to the Inventors of Prozac.
Last but by no means least as this thesis would not have happened without him. **Daniel Browne**, for his patience love and support, who fed me and loved me, and who I’m sure is excited to see who his girlfriend is when she’s not shackled to a thesis. Thanks for fixing all the programs I had to install and helping me cut seeds late at night and calming all my fears.

Also thank you to my fears, my demons, my anxiety. I learnt were you help me and where you cripple me and how to keep you in your place.

Abstract

In this thesis I determine the effect of population decline on reproduction for two critically endangered African cedars. *Widdringtonia whytei* (Mulanje cedar) endemic to Mt Mulanje, in Malawi and *Widdringtonia cedarbergensis* (Clanwilliam cedar) endemic to the Cedarberg Mountains in the Western Cape Province of South Africa.

Populations of both species have declined dramatically in the recent past and both show evidence of insufficient recruitment. Recent research has shown that a reduction in population density may limit pollen dispersal, reduce seed viability and increase self-pollination rates, causing an inbreeding depression, resulting in less fit offspring. Based on this research, I hypothesise that pollen transfer in *Widdringtonia* is distance-dependant and therefore populations with greater distances to the nearest adult neighbour will have lower seed viability, due to pollination failure. I also hypothesise, that trees which receive less outcross pollen due to distance-dependant pollination will have higher rates of self-pollination. I further hypothesise that seed viability may also increase with increased soil nutrients and more amenable climate differences linked to changes in altitude.

To test these hypotheses, I determine the extent to which reduced population density has resulted in a decline in viable seed using germination experiments, followed by cut tests and tetrazolium chloride tests. For *W. cedarbergensis* seedling survival was determined in a greenhouse and self-pollination was assessed with ISSR markers, using DNA extracted from parent and offspring.

My results show that seed viability for both *W. cedarbergensis* and *W. whytei* is not significantly correlated with distance to nearest-neighbour, altitude or soil nitrogen, carbon or phosphorus. These results suggest that current population densities are not effecting the reproduction either of *W. whytei* or *W. cedarbergensis*. My results for population genetics show relatively low levels of genetic variation in *W. cedarbergensis* typical of endangered and endemic species. The genetic differentiation between populations is low, suggesting that pollen flow between populations is adequate and populations are not genetically isolated. I conclude that there is no evidence that population decline is causing any noticeable limitations on pollen transfer and reproduction in *Widdringtonia.*

Chapter 1

Introduction

The genus *Widdringtonia* Endl. (Cupressaceae), contains four species, all of which are endemic to southern Africa (Coates Palgrave 2002). Of these, *W. whytei* Rendle is restricted to Mount Mulanje in Malawi, *W. cedarbergensis* Marsh to the Cedarberg Mountains, 200 km north of Cape Town in South Africa, and *W. schwarzi* (L.) Powrie to Baviaanskloof, near Willowmore, South Africa (Manders 1986b; Pauw and Linder 1997; Coates Palgrave 2002). The fourth species, *W. nodiflora* (Marloth) Mast. is a shrubby, multi-stemmed resprouter commonly found throughout the sub-region in fire-prone vegetation types, such as grassland and fynbos. While the first three species are all large trees, *W. nodiflora* may only reach tree size in the absence of fire (Pauw and Linder 1997; Coates Palgrave 2002). Of these four species, both *W. cedarbergensis* and *W. whytei* are listed as rare and endangered, facing extinction in the wild, while *W. nodiflora* and *W. schwarzi* are both viable species (Raimondo *et al* 2009). This study focuses on *W. whytei* and *W. cedarbergensis*, as it is these two species that are critically endangered (IUCN 2015).

Widdringtonia cedarbergensis, also known as the Clanwilliam cedar, is listed as a protected species and, as mentioned above, is on the verge of extinction (Raimondo *et al* 2009). Historically, the tree was extensively logged, as it was the only wood source in the area (Lückhoff 1971). The first protective measures were put in place as early as 1879, with attempts made to ban harvesting, control livestock grazing and limit fire (Lückhoff 1971). The Cedarberg Wilderness area has been managed as a reserve since 1973, with efforts made to replant seedlings to boost numbers in the wild (Manders 1986a; Muir 2002; Mustart 2013). Despite these protective measures, numbers for this species have continued to decline (Lückhoff 1971; Manders 1986a; Higgins *et al* 2001; White 2013). Although there is much debate as to whether the decline of *W. cedarbergensis* is part of a long-term trend, anthropogenic in cause, or an additive combination of long-term change being accelerated by anthropogenic disturbances, the species is becoming increasingly fragmented, with no improvement from its critically endangered status (Manders 1987b; Sudgen and Meadows 1990; Higgins 2001; White 2013). In less than 30 years, with conservation measures in place, some populations lost as much as 92% of trees between 1977 and 2003 (Fox 2003).

The Cedarberg Mountains fall within the Fynbos biome in the Cape Floristic Region, which is well known for its high plant species diversity and endemism. The 90 000 km² region boasts over 9000 plant species, 68% of which are endemic (Goldblatt 1978; Goldblatt and

Manning 2002). Fynbos is a fire adapted vegetation type and, anomalously, *W. cedarbergensis* is extremely fire sensitive (White 2013; Kruger 1979). Seedlings and most adults of *W. cedarbergensis* are unable to survive direct fire (Manders 1986a) or proximity to attendant heat (pers obs).

Widdringtonia cedarbergensis typically favours an altitude between 800 and 1650 m (Manders 1986a; Higgins *et al* 2001), but recent work using repeat photography demonstrate increasing mortality at lower altitudes (White 2013). This research suggests that this increase in mortality may be associated with increasing temperatures due to climate change as well as an increase in fire frequency. As much as 54% of the vegetation in the Cedarberg burns with a fire return interval of less than 15 years, with 74% burning before it is 20 years old (Southey 2009). Manders (1987b) suggested that a fire return interval of 15-20 years is necessary for the survival of the cedars. This means that more than half of the Cedarberg will burn before it meets the lower fire return interval suggested by Manders (1987b). These changes in the fire return interval are likely a consequence of anthropogenic climate change, resulting in climate conditions associated with fire ignition becoming more frequent (Southey 2009). As *W. cedarbergensis* takes 12-40 years to reach maturity and produce significant amount of seed (Andrag 1977), these very short fire return intervals are likely to result in trees being destroyed by fire before reaching maturity, causing a decline in the species. In addition to being extremely fire sensitive, *W. cedarbergensis* displays only weak serotiny (Botha 1990), resulting in very few seeds available for release after a fire for post-burn germination (Botha 1990).

Widdringtonia whytei, also known as the Mulanje cedar, occurs in forest patches among grassland, on the plateau of Mount Mulanje, Malawi, between 1500 – 2200 m above sea level (Chapman 1995). Species diversity on the mountain is relatively high, with more than 1300 species recorded, 5.3% of which are endemic in an area of only 650 km^2 (Strugnell 2002; Bayliss 2007). Mount Mulanje was declared a reserve in 1927 (Strugnell 2002). In 1984, the Mulanje cedar was made the national tree of Malawi (Makungwa 2004), and in 1991 the Mulanje Mountain Conservation Trust was formed to implement conservation strategies on the mountain (Strugnell 2002). These strategies include ranger patrols to prevent poaching, and a cedar re-planting program (Makungwa 2004). Despite these protective measures, numbers of *W. whytei* have continued to decline by more than 2.27% in the decade between 1994 and 2004, with numbers continuing to decrease thereafter (Makungwa 2004, pers obs).

It has been suggested that one of the reasons for the decline in *W.whytei* is fire (Chapman 1995, Bayliss *et al* 2007, Chanyenga *et al* 2011b). The grasslands of Mount Mulanje experience frequent fires, which are often anthropogenic in origin (Chapman 1995; Bayliss 2007). Poachers light fires to flush out game on the mountain, or to avoid being apprehended by authorities (Bayliss 2007; pers comm with Forestry rangers). Fires may also move up the mountain from the surrounding villages (Chapman 1995). These fires spread unchecked, and if the forest is breached, can eradicate *W.whytei* seedlings and weaken or even kill the exceptionally flammable mature trees (Chapman 1995; Bayliss 2007). It has, however, been suggested by Chapman (1995) that a fire return interval of around 100 years opens the canopy of the forest, and is therefore important for the regeneration of the species. Seedlings are frequently found on the edge of forest patches, and an open canopy is important for their regeneration (Chapman 1995). With 325 fires recorded on the mountain between 2001 and 2006, many of these penetrating into the forest, such a long fire return interval is unlikely to occur (Chanyenga *et al* 2011b; Makungwa 2004).

Climate change and the presence of the alien aphid, *Cinara cupressi*, are also listed as potential threats to *W. whytei*, but the most noticeable threat to these trees is illegal logging (Chapman 1995; Makungwa 2004; Bayliss *et al* 2007). The wood of *Widdringtonia whytei* is extremely valuable, as it is pliable, durable, insect-resistant and aromatic (Chapman 1995). The tree was extensively harvested by former Forestry Departments for over 100 years, and is still logged illegally by local poachers (Makungwa 2004; Bayliss *et al* 2007; pers obs 2012). The removal of these slow-growing trees is considerable, often with whole stands being destroyed (Bayliss 2007; Chapman 1995; pers obs). The remaining clusters of cedars are now found primarily at the bottom of inaccessible gorges, because these areas provide protection from both fire and illegal harvesting (Makungwa 2004).

Widdringtonia whytei and *W. cedarbergensis* are both wind-pollinated species that are showing increasing rates of fragmentation and reduced reproduction (Coates Palgrave 2002; Chapman 1995; Higgins *et al* 2001; Thomas and Bond 1997; Chanyenga *et al* 2011b). In addition to the obvious reduction in abundance, fragmentation and population decline may also lead to a decreased probability of pollination with outcross pollen, reduced seed set, obligatory inbreeding, and self-pollination (Mimura and Aitken 2007; Farris and Mitton 1984; Allison 1990; Knapp *et al* 2001). This is because the distance between plants and plant density are limiting factors for pollen transfer in wind-pollinated species (Allison 1990; Knapp *et al* 2001). A pollen cloud is the accumulative quantity of pollen in the atmosphere,

and is usually contributed to by several neighbouring trees or the entire population (Allison 1990; Sork *et al* 2002; Mimura and Aitken 2007). Because a pollen cloud is not always homogenous across a population, a reduction in the number of adults, or an increase in the distance between adults, reduces the amount of available pollen transported by the wind. This decreases fertilization and the production of viable seed (Mimura and Aitken 2007; Sork *et al* 2002; Rajora *et al* 2002; Allison 1990; Knapp *et al* 2001).

Higgins *et al* (2001) showed that, prior to 1985, the overall size-class distribution of *W. cedarbergensis* was a typical reverse J-shaped curve, with numerous juveniles and progressively fewer adults. Despite the large number of juveniles, a very low percentage of the trees was in the smallest size classes, suggesting poor initial recruitment (Higgins *et al* 2001). The overall size-class distribution for *W. whytei*, in the decade from 1994-2004, deteriorated from a typical reverse J-shaped curve to a normal distribution, with the lowest density of trees found in the smallest size class with diameters between 5 and 15 cm (Lawrence *et al* 1994; Makungwa 2004). It has been proposed that a distribution such as this, with few juveniles, demonstrates a population decline (Condit *et al* 1998). The current size-class distribution of *W. whytei* is similar to that of *W. cedarbergensis* but, lacking an historical assessment of size-class distribution for *W. cedarbergensis,* it is difficult to determine if this is typical of the species. In light of the rapid decline of both species, however, it is worth investigating if there is a problem with recruitment, as low recruitment can be the result of population fragmentation and increased distance to the nearest-neighbour (Farris and Mitton 1984; Knapp *et al* 2001). This increased distance to nearest neighbour could cause outcross pollination to become an increasingly rare event while also increasing the incidence of self-pollination, both of which could result in reduced seed viability (Allison 1990; Sork et al 2002; Mosseler *et al* 2000; Rajora *et al* 2002).

While germination rates of up to 51% for *W. cedarbergensis* and an average seed viability of 23% for *W. whytei* have been demonstrated (Manders 1987a; Chanyenga 2011a), there has been no research on the effect of distance to nearest neighbour on the seed viability for either species. For *W. whytei* large forest patches have the highest seed viability, followed by small patches and isolated trees, with medium-sized patches having the lowest seed viability (Chanyenga *et al* 2011a). This distribution in seed viability may be a function of a single tree's proximity to the nearest potential pollen donor, or the population density, rather than the size of the population as a whole.

Decreases in seed viability may also be caused by environmental factors that are unrelated to population density and self-pollination. The Cedarberg has very nutrient-poor, acidic, sandstone-derived soil and Mount Mulanje has iron-rich, acidic, nutrient poor, granite-derived soils that have low proportions of nitrogen and phosphorous (Young and Stephen 1965; Chapman and White 1970; Taylor 1996; Quick and Eckardt 2015). The low-nutrient soils of the Cedarberg are typical of fynbos soils, which are low in both nitrogen and phosphorous, these being cycled back into the soil through periodic fires (Manning 2007). It has been suggested that species distribution in fynbos is related to the spatial variation in available nitrogen and phosphorus (Richards *et al* 1997). Some plant species show evidence of reduced seed set when nutrient -limited (Renison *et al* 2004; Wallace and O'Dowd 1989; Campbell and Halama 1993). If some *W. whytei* and *W. cedarbergensis* sites have less available nutrients than others then this could limit the production of viable seed at those sites. This would be an important consideration for replanting programs.

Seed viability has also been shown to decrease with increased altitude in some conifers (Burrows and Allen 1991; Holm 1994; Johnstone *et al* 2009). There is typically an increase in rainfall and decrease in temperature with an increase in altitude (Barry and Chorley 2010). This may suggest that seed viability is likely to be influenced by either increasing rainfall, or decreasing temperature, with increasing altitude. There is also some evidence that the survival of *W. cedarbergensis* is greater at higher altitudes and lower temperatures and it has been suggested that this may be related to increased temperatures at lower altitude as a result of anthropogenic climate change (White 2013). If adults favour lower temperatures, and anthropogenic climate change increases temperatures at lower altitudes, temperatures at higher altitudes may become more amenable to seed production, resulting in higher seed viability at higher altitudes. There have been no previous studies on the relationship between altitude and seed viability for either *W. whytei* or *W. cedarbergensis.*

A reduced number of contributors to the local pollen cloud may also subsequently increase the chance of self-pollination (Mimura and Aitken 2007). Self-pollination, in addition to other forms of inbreeding, can cause inbreeding depression, where reduced genetic variability results in reduced fitness, which may be evident in lower proportions of viable seed and lowered relative fitness in offspring (Charlesworth and Charlesworth 1987; Rajora *et al* 2000; O'Connell and Ritland 2005; Robledo-Arnuncio *et al* 2004; Mimura and Aitken 2007; Mosseler *et al* 2000). While there are currently no known studies of self-pollination or genetic variability in *W. whytei*, there is some evidence of inbreeding in *W. cedarbergensis*

and it has been suggested that this is a result of declining population density and a corresponding increase in self-pollination (Thomas and Bond (1997). The poor recruitment displayed in these species may be a result of declining populations causing outcross-pollination failure and increased self-pollination, resulting in low seed viability and decreased offspring fitness and survival. *W. whytei* germinates readily, but does not establish easily (Chapman 1995, Bayliss *et al* 2007). *W. cedarbergensis* also germinates easily (Higgins *et al* 2001), but drought and herbivory are known to greatly diminish establishment (Manders and Botha 1989; Higgins *et al* 1989). This lack of seedling vigour in both species may well be an indication of an inbreeding depression. This can be tested indirectly, by determining if there is a correlation between trees with low seed viability and low seedling survival. Inbreeding can also be tested directly, by means of genetic analysis.

There is increasing evidence suggesting that *W. whytei* and *W. cedarbergensis* populations are declining (Manders 1986a and 1987; Lawrence *et al* 1994; Chapman 1995; Higgins *et al* 2001; Bayliss *et al* 2007, Makungwa 2004; White 2013). If conservation efforts are to be successful, it is imperative that we gain an understanding of the reproduction, recruitment and establishment, of both *W. whytei* and *W. cedarbergensis*, as well as how these may be affected by declining populations. In this thesis I ask what the impact of such a decline is on the reproductive success of both *W. whytei* and *W. cedarbergensis*. I hypothesize that increased fragmentation of populations and decline in the number of adult trees will result in a reduction in reproductive success, primarily through a reduction in outcross pollination success. I further hypothesize that the reduction in pollination success will result in obligatory self-pollination and an inbreeding depression. I investigate this hypothesis in two data chapters.

In Chapter 2, I determine the seed viability for each species and the environmental factors that influence seed viability as well as seedling survival for *W. cedarbergensis*. I hypothesise that trees which have a greater distance to the nearest adult neighbour will have lower seed viability and seedling survival rates, due to increased difficulty of pollen transfer and increased inbreeding depression. I examine this hypothesis in a study where I germinate seeds of both species in a phytotron where I control temperature, and then determine seedling survival in a greenhouse for six months after germination. I hypothesize that sites with higher availability of soil nutrients will have higher levels of seed viability. I also hypothesize that seed viability will increase with altitude as conditions at higher altitudes become more amenable to reproduction than those at lower altitudes, as a result of anthropogenic climate

change. I examine this hypothesis by determining soil nitrogen, phosphorus and carbon, as well as altitude, at each site and relating these to seed viability determined for each site from my germination experiment.

In Chapter 3, I determine the genetic evidence for self-pollination in *W. cedarbergensis*. Here I hypothesise that *W. cedarbergensis* will show high levels of self-pollination with low seed viability and low seedling survival. I also hypothesise that a higher proportion of seedlings that died in the first 6 months after germination would be self-pollinated than those seedlings surviving the first six months. I determine this using ISSRs. The degree of self-pollination can be correlated with viability and seedling survival to determine the effects, if any, of inbreeding.

In Chapter 4, my synthesis chapter, I explore my results relative to my hypothesis and make statements on the impacts of population decline on reproduction and recruitment of *W. whytei* and *W. cedarbergensis*. I conclude by making recommendations as to how my results may be interpreted to help in the conservation and management of both of these iconic tree species.

Chapter 2

Population spatial structure, seed viability, and seedling survival

Introduction

Plant conservation is becoming increasingly concerned with pollen-limitation in fragmented and reduced populations (Sork *et al* 2002; Mosseler *et al* 2000; Rajora *et al* 2002; Fady *et al* 2008). A lack of pollen can affect both the reproductive yield in self-incompatible plants, and hinder genetic transfer within and between populations (Mimura and Aitken 2007; Dudash and Fenster 2000; Mitton 1992; Allison 1990). In many animal-pollinated plants, seed set is negatively correlated with population density, as pollinators must travel greater distances between plants (Cunningham 2000; Ghazoul *et al* 1998; Silander 1978; Caraballo-Ortiz *et al* 2011). It has been suggested that anemophily (wind pollination) can more easily overcome this spatial barrier, as the pollen may travel greater distances without being dependent on the behaviour and needs of another organism (Wang *et al* 2011). There are, however, several studies that contradict this hypothesis as wind-pollinated plants have shown low seed set and increased inbreeding when populations become fragmented, due to a decrease in the amount of available pollen (Aizen and Feinsinger 1994; Berry and Calvo 1989). Such pollen limitation has been demonstrated for several species of birch (Holm 1994) and oak (Knapp *et al* 2001; Sork *et al* 2002; Jump and Peñuelas 2006), as well as several conifers (Arista and Talvera 1996; Farris and Mitton 1984; Robledo-Arnuncio *et al* 2004).

A number of studies have demonstrated that a reduction of available pollen may reduce ovule fertilization, resulting in a reduction in seed set due to pollination failure (Knapp *et al* 2001; Allison 1990; Smith *et al* 1990). Such pollination failure has been demonstrated in California, USA, where populations of *Quercus douglasii* showed a reduction in acorn production with decreasing numbers of pollen-producing neighbours (Knapp *et al* 2001), and in Colorado, USA, where *Pinus contorta* showed a significant correlation between the density of atmospheric pollen and seed set (Smith *et al* 1990).

Widdringtonia whytei and *W. cedarbergensis* are both experiencing population decline and there is a risk that this may also be decreasing their reproduction through pollen limitation. Seed viability for *W. whytei* is around 23% and, depending on substrate, *W. cedarbergensis* has between 8 and 51% germination (Chanyenga *et al* 2011a; Manders 1987a). Reported values for seed viability or percentage filled seed in conifers are extremely variable. For example, *Pinus sylvestris* has a seed viability of 98% (Granström 1987), and *Thuja plicata* a viability of 71 to 87% (O'Connell and Ritland 2005). These figures are considerably higher

than those previously measured for *Widdringtonia* (Manders 1987a; Chanyenga *et al* 2011a), however, several conifers show much lower values than those for *Widdringtonia*. *Juniperus oxycedrus* subsp. *macrocarpa*, has seed viability of 6 to 12% (Juan et al 2003) while *Larix occidentalis* has values between 9 and 30% (Stoehr 2000). Although it is not known if the values for seed viability reported for *W. whytei* and *W. cedarbergensis* are similar to values prior to population decline, there is some evidence to suggest that seed viability for *W. whytei* is related to population size. Seed viability for *W. whytei* is highest in larger forests patches, but seed viability in medium-sized patches is lower than in small patches and isolated trees (Chanyenga *et al* 2011a). Considering these results and the effect of population reduction on seed viability in other species, it is important to test if distance to nearest- neighbour, as a measure of population density, is restricting pollen movement and reducing seed viability in *W. cedarbergensis* or *W. whytei*.

If low seed viability is caused by a decrease in pollination events in *Widdringtonia*, trees experiencing low pollen input, and subsequently low seed viability, may also receive higher proportions of self-pollen. This is because in the absence of large amounts of outcross pollen there are higher proportions of self-pollen available in the pollen cloud (Mimura and Aitken 2007). Gymnosperms are wind pollinated, often monoecious, and commonly lack mechanisms to avoid self-pollination (Sorensen 1982). Self-pollination can result in inbreeding depression, which is a decline in fitness as a result of increased homozygosity of lethal or semi-lethal alleles (Charlesworth and Charlesworth 1987; Mosseler *et al* 2000; Lowe *et al* 2005). Inbreeding depression can result in high proportions of empty seed and loss of fitness in offspring (Rajora *et al* 2000; O'Connell and Ritland 2005). For example, *Picea rubens,* in Canada, shows a positive relationship between seedling height and outcrossing rates (Mosseler *et al* 2000) and *Pinus strobus*, also in Canada, shows decreased outcrossing rates with decreased population density (Rajora *et al* 2002). If self-pollination is occurring in *Widdringtonia* as a result of reduced population density, this could explain the poor establishment for *W. cedarbergensis* (Higgins *et al* 2001). Here, I test the relationship between seed viability and seedling survival for *W. cedarbergensis,* to determine if populations with low tree densities also have low seed viability, and high rates of self-pollination, with higher mortality rates in offspring, as a result of reduced seedling fitness.

The soil on Mount Mulanje is generally nutrient-poor, granite-derived, iron-rich and acidic (Young and Stephen 1965; Chapman and White 1970) while fynbos, the dominant vegetation in the Cedarberg, grows on nutrient poor acid sandstone-derived soils (Moll and Jarmaan

1984; Van Wilgen 1982; Manning 2007). Although soil nutrients have been demonstrated to affect seed production in species such as *Banksia marginata* (Vaughton and Ramsey 1998), it is not known if nutrient availability plays a role in seed viability for either *W. whytei* or *W. cedarbergensis.* I test this by determining if there is a relationship between seed viability and soil nitrogen, phosphorous and carbon.

Precipitation tends to increase while temperature decreases with increased in altitude (Barry and Chorley 2010). These changes in rainfall and temperature may be responsible for the decrease in seed viability with an increase in altitude seen in a few species of conifers (Burrows and Allen 1991; Holm 1994; Johnstone *et al* 2009). *W. cedarbergensis* shows evidence of fewer deaths at higher altitudes where there are cooler temperatures than at lower altitudes where the temperatures are higher (White 2013). The temperatures at lower altitudes may also be increasing as a result of anthropogenic climate change, forcing the climatic envelope for the species upslope (Haplin 1997; Midgley *et al* 2003; Hannah *et al* 2005). If climate conditions at higher altitudes are becoming more suitable for *W. cedarbergensis* with time then the climate may also be more suitable for reproduction. I therefore determine the relationship between seed viability and altitude for *W. cedarbergensis* and *W. whytei*.

In this Chapter, I investigate the seed viability for both *W. cedarbergensis* and *W. whytei* and how this may relate to the spatial structure of the populations of each of these species. I hypothesise that pollen transfer is distance-dependent and therefore populations that have a greater average distance to nearest adult neighbour will have lower seed viability as a result of outcross-pollination failure. I also investigate whether seed set is linked to environmental variables such as altitude and soil nitrogen, phosphorous and carbon. I hypothesise that seed viability will increase with an increase in altitude and soil nutrients. I further hypothesise that there will be a correlation between seed viability and seedling survival, as individuals that are pollen-limited will show higher rates of self-pollination, resulting in higher mortality rates, as a result of less fit offspring.

Methods

Study sites

My two study sites are on Mount Mulanje in Malawi and the Cedarberg Mountains in South Africa.

Mount Mulanje, (Widdringtoni whytei)

Mount Mulanje is a 680 km² granite batholith in southern Malawi, 200 km east of the city of Blantyre (Beard 1997). The plateau is 1800 m above sea level while the highest peak, Sapitwa, is 3002 m above sea level (Beard 1997). The majority of *W. whytei* trees occur on the plateau between 1500 and 2200 m above sea level (Chapman 1995). Mount Mulanje receives between 2000 and 3000 mm rainfall during the wet summer which lasts from November to April (Chanyenga *et al* 2012; Curran *et al* 2012). During the relatively dry cold winter season, between May and September, the mountain may receive up to 21% of its annual precipitation in the form of mists and light rain (Dowsett-Lemaire 1988; Curran *et al* 2012). Temperatures can range between 28° C in the wet season and 5° C in the dry season, with occasional frost (Chapman and White 1970; Chanyenga *et al* 2012). The soils on the plateau are acidic and termed ferallitic latosols which are characteristically rich in iron and aluminium. The soil in the forest patches are also nutrient-poor with low nitrogen and phosphorous (Young and Stephen 1965; Chapman and White 1970). The vegetation on Mount Mulanje is predominantly grassland, interspersed with patches of afromontane forest in areas protected from fire (Beard 1997). It is in these forest patches that *Widdringtonia whytei* may be found (Chapman 1995).

Some of the endemic or near-endemic graminoids which can be found on the mountain are *Tetraria mlanjensis* (Cyperaceae), *Eragrostis fastigiata*, *Digitaria trinervis*, *Rytidosperma davyi*, *Alloeochaete oreogana* and *A. geniculata* (Poaceae) (Strugnell 2002). *W. whytei* may occur in pure stands (Chapman 1995) or as the only canopy emergent in afromontane forest composed of species such as *Ilex mitis*, *Podocarpus latifolius, Kiggelaria africana*, *Pittosporum viridiflorum* and *Rapanea melanophloeos* (Chapman and White 1970; Dowsett-Lemaire 1988). The natural extent of these forests is limited by regular grass- fuelled fires that create sharp boundaries between forest patches and the surrounding grassland (Beard 1997; Chapman 1995).

Sample collections were made from the following 6 locations on Mount Mulanje between the 9th and the 19th October 2012. All populations' sampled are considered to be naturally occurring and not planted.

- **Sombani** (S15°53’30.9’’ E35°42’37’’): Sombani is a closed canopy forest, towards the edge of the plateau, with many adult and a few juvenile trees. The stand has a mean density of 99 stems ha^{-1} (Bayliss *et al* 2007).
- **Nomoso Pools** (S15°54’56.7’’ E35°41’36.6): This site extends up a slope away from a river, the lower section has a partially open canopy with thick impenetrable undergrowth. The undergrowth becomes less dense and the trees more sparse, moving uphill away from the river.
- **Madzeka** (S15°57’35.3’’ E35°41’31.6’’): This patch grows on a steep incline with dense undergrowth. The canopy is closed in parts but not in others. The stand has a mean density of 49 stems ha^{-1} (Bayliss *et al* 2007).
- **Chinzama** (S15°53’54.3’’ E35°39’10.4’’): This population is on a relatively gentle slope and has a dense undergrowth, with a few trees growing away from the main patch. The mean stand density for this site is 77 stems ha^{-1} (Bayliss *et al* 2007).
- **Lichenya-Kangazani** (S15°57’51.6’’ E35°32’50.3’’): This population is on a steep slope, with a closed canopy and the cedars emerging above the canopy. Here the undergrowth is sparse. The two Lichenya stands have an average density of 56 stems ha^{-1}(Bayliss *et al* 2007).
- **Lichenya- Limbe** (S15°58’02.7’’ E35°33’12.0’’): This population is near a river, and has fairly dense undergrowth and an open canopy.

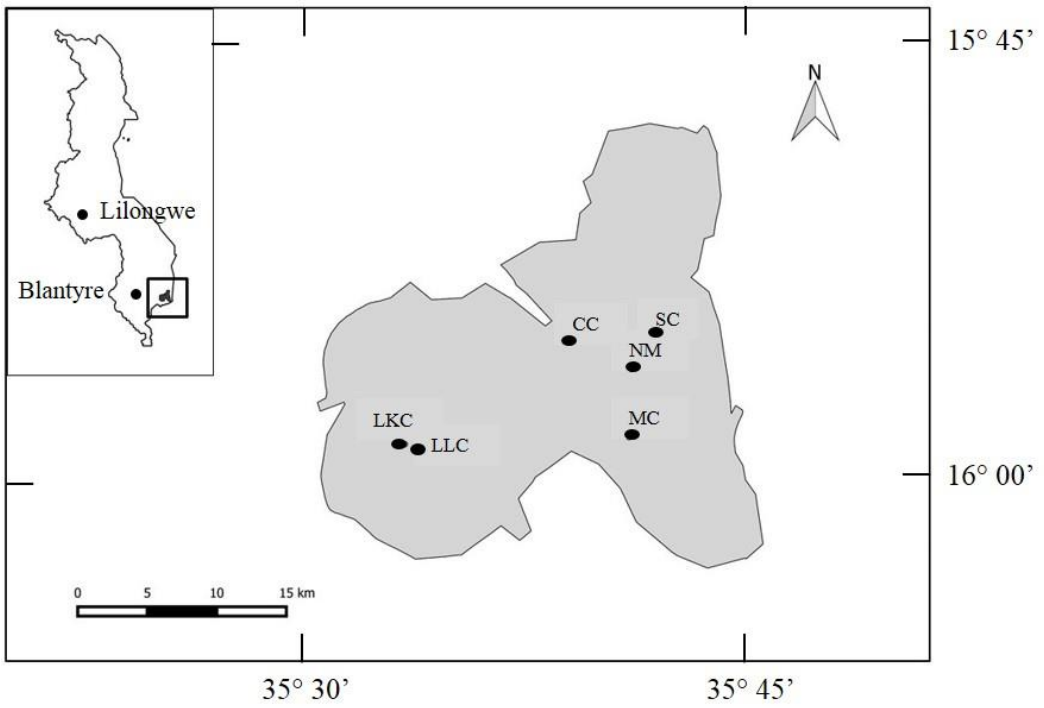

Figure 1.1: Map showing the sites sampled on Mt Mulanje, with an inset showing the mountains position, east of Blantyre in Malawi. A - Sombani, B - Nomoso Pools, C- Madzeka, D Chinzama, E – Lichenya-Kangazani and F- Lichenya-Limbe.

The Cedarberg Mountains (Widdringtonia cedarbergensis)

Widdringtonia cedarbergensis has a patchy distribution over the Cedarberg Mountains in the Western Cape Province, South Africa, growing at an altitude between 900 and 1400 m above sea level (February et al 2007). These mountains have a Mediterranean climate with warm, dry summers and cool, wet winters. Annual rainfall between 1994 and 2004 at Welbedacht was 751 mm (February et al 2007). Summer temperatures regularly reach 25-30°C and may rise to 40°C with winter temperatures frequently below freezing at night (Taylor 1996). The dominant vegetation type is fynbos, a sclerophyllous, fire-prone shrubland, characterised by the families Proteaceae, Ericaceae and Restionaceae and growing on nutrient-poor acid sandstone-derived soils (Moll and Jarmaan 1984; Taylor 1996; Van Wilgen 1982; Manning 2007). While *W. cedarbergensis* is the only tree endemic to the Cedarberg, there are several tall shrubs such as *Protea nitida* and the endemic *Leucospermum reflexum*. Other endemics include the low shrub *Erica cedarbergensis* and the graminoid *Ficinia cedarbergensis* (Mucina and Rutherford 2006).

Sample collections were made from the following 5 locations in the Cedarberg between the 7th and the 15th of March 2013;

- **Sneeuberg** (S32°29'25.9'' E19°10'23.1''): This is a naturally occurring population and has many adult trees as well as several juveniles. At the time of collection a recent fire had killed several adults. Collections were made only from trees unaffected by the fire.
- **Welbedacht** (S32°24'29.98'' E19°10'44.72''): This is a naturally occurring population which grows in amongst large boulders, on a steep slope. It has many mature trees but few juveniles. Due to the difficulty of finding and obtaining cones from this site, only 5 adults were sampled.
- **Heuningvlei –vlakte** (S32°12'59.29'' E19°05'38.43''*): Vlakte* is Afrikaans for flats and this plantation was planted in the late 1800's near the village of Heuningvlei on a flat plane of deep sand. The trees grow quite close together with little to no vegetation below the canopy.
- **Heuningvlei – mountains** (S32°11'46.21'' E19.04'36.9''): This population is scattered on the mountain slopes and koppies above the village of Heuningvlei. Some of these individuals were planted but many have established there naturally.
- **De Rif** (S32°26'20.8'' E19°13'54.8''): This is a plantation that was planted in the late 1800's below a steep cliff at an old farmstead. The trees are close together here, forming closed canopies in some parts and some trees exceed 10 m in height.

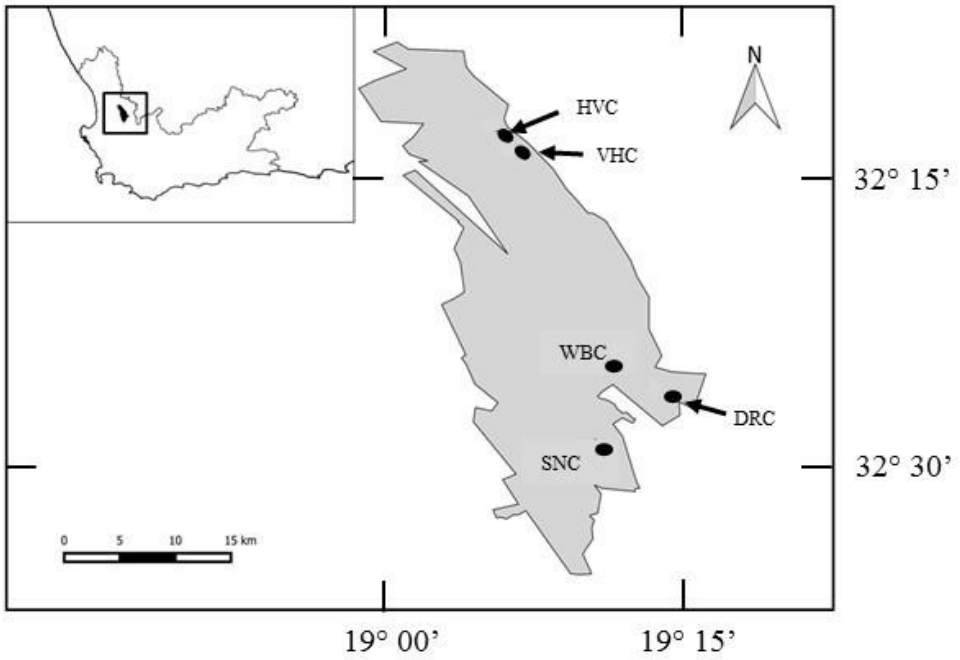

Figure 1.2: map of the sites sampled in the Cedarberg Wilderness Area with an inset of its position within South Africa and the Western Cape. A – Sneeuberg, B- Welbedacht, C – Heuningvlei – mountains, D-De Rif and E – Heuningvlei –vlakte.

Field sampling

Ten cones were harvested per tree for six trees at the six sites on Mount Mulanje and five sites in the Cedarberg. I only sampled five trees at the Welbedacht site in the Cedarberg, because the trees were growing on steep cliffs and it was too dangerous. From each parent tree the distance to the nearest adult neighbour was measured if less than 15 m, and estimated by eye by the same person, if greater than 15 m. For *W. cedarbergensis*, an average was taken for the distance to the four nearest neighbours. This gives a measure of population density for *W. cedarbergensis*. For *W. whytei*, the measure of population density comes from only one nearest neighbour per tree, averaged for the whole population. While there is indeed a predominant wind direction at both of my study sites I rationalise that wind is turbulent and eddies in rocky and mountainous terrain. Despite prevailing wind directions, one would expect the very varied topography of my study sites to cause eddies negating the effects of a prevailing wind direction, thereby enabling the use of nearest neighbour in any particular direction.

To test the relationship between soil nutrients and seed viability for *W. cedarbergensis*, six soil samples were collected from the top 10 cm of soil at each site. Three of the six samples were collected under a parent tree and the other three were collected away from the tree.

(except for Welbedacht and De Rif where 5 samples, 3 under and 2 away, and Heuningvlei-mountains, where 2 under and 3 away samples, were collected).Three samples were collected per site for *W. whytei* and, by default, these were taken from under trees as *W. whytei* predominantly grows in closed canopy forest. Soil was tightly sealed in polyurethane bags until processed in the laboratory at the University of Cape Town.

Seed viability

Seeds from 10 cones from each of the 6 trees per site, except for the Welbedacht site where 5 trees, were sampled, were used for germination experiments. In a few instances some of the cones had a majority of seed which was not yet ripe and these cones were discarded as they would have given a skewed measure of viability. No tree had less than 5 fully ripe cones (see Appendix 1 and 2). In the laboratory at the University of Cape Town, cones were opened by air drying in the shade. The few that did not open were cracked with a light tap from a small hammer. For *W. whytei* there was an average of 10.1 seeds per cone and for *W. cedarbergensis* this was 10.6 seeds per cone. This included all seeds present in the cone irrespective of their category (for seed categories see Appendix 3). All seeds from each of the 10 cones per tree were placed on a 1% solution of agar in petri dishes as per the ISTA (International Seed Testing Association) standards (ISTA, 2003), with the addition of a loosely-fitted glass cover to limit mould infiltration, and slow the rate at which the agar dried. To maintain sterility the agar was autoclaved prior to pouring into the petri dishes. The seeds were placed in a phytotron where the day/night temperatures for *W. cedarbergensis* were set at 19/20 ± 2 º C and 20/20 ± 2 º C for *W. whytei* with 12 hours of daylight and 12 hours of night. These temperature regimes were based on those which Gondwe (2010) found to be most successful for germinating *W. whytei*. Light was provided by 150 W sodium, 250 W/D metalhalide and 100 W/230 V incandescent light bulbs.

The number of germinations were visually ascertained and recorded daily until there was no germination for two weeks. Seeds that had not germinated were examined with a cut test to determine if these were empty, mouldy/rotten or filled and fresh (ISTA, 2003). Fresh seeds were subjected to a tetrazolium chloride (TZ) test to determine viability, using the ISTA protocol (ISTA, 2003), with the adjustment that seeds were left in the solution for 48 hours at room temperature. This was a longer time and a lower temperature than the 6-18 hrs at 30-35º C suggested by Miller (2010) for the Cupressaceae, as there was concern that these may contain oils which would impede the absorption of tetrazolium chloride. Seeds were cut transversally a third of the way down from the apex and, where possible, as much of the seed

coat as possible was removed. The percentage seed viability was calculated using the total number of viable seeds (germinated seed and viable ungerminated seeds) and the total of all seeds which included empty, rotten/mouldy seed, and full fresh non-viable seed (see Appendix 3). 'Full, non-viable seed' comprised of rotten/mouldy seed and full, fresh non-viable seed. In some species small aborted seeds are common at the top and bottom of the cone (Owens and Fernando 2007) so seeds that were less than 0.063cm^2 were excluded from calculations for *W. cedarbergensis*. This cutoff size was chosen as it was considerably smaller than the smallest seed to germinate.

Soil analysis

Soil samples were transported in backpacks on the mountain and on the flight back to Cape Town and then stored in a 10° C cold room until processing. Prior to analysis, the soil was sieved with a 1 mm mesh sieve to remove roots and leaf litter. The soil was then dried in an oven at 70-80°C for at least 48 hours (Scientific Series 2000, model 278, South Africa). Plant available phosphorous was determined at the Institute for Plant production, Department of Agriculture, Elsenburg, where inductively coupled plasma analysis (iCAP 6500 ICP, ThermoFisher Scientific, MA, USA) was performed on soil extracts, obtained from a 1% citric acid solution according to the methods of Dyer (1894) as modified by Allen (1940).

I determined percentages of total soil carbon and nitrogen, using a Flash 1112 Elemental Analyser coupled with a Delta Plus XP Isotope Ratio Mass Spectrometer with a Conflo III gas control unit (Thermo electron, Bremen, Germany) at the University of Cape Town. In-house standards, calibrated against the IAEA (International Atomic Energy Agency) standards, were used to calibrate the results relative to atmospheric N_2 for nitrogen and Pee Dee Belemnite for carbon.

Altitude

In order to determine the relationship between seed viability and altitude, altitude was recorded for each site using a GPS (Garmin Etrex 10, USA).

Seedling survival

I only determined seedling survival for *W. cedarbergensis* and not for *W. whytei.* Within approximately two weeks of germination, seedlings were placed into seedling trays filled with potting soil (SuperGrow Potting Soil, Stanler Farms) in a phytotron under the same conditions in which they had germinated before being moved into a green house, at ambient

temperatures. For *W. cedarbegensis* there was a total of 1247 germinations across all the sites, ranging between 289 (Heuningvlei –Mountains) and 383 (Welbedacht) germinations. The number of plants that died each day were determined for six months after the first month of germination and the death rate calculated as a percentage of original germination. *W. cedarbergensis* seedlings were divided into three groups according to the stage of growth they were at the time of death. Stage 1 = from germination to the growth of the first two leaves resulting from the cotyledons. Stage 2 = seedlings with secondary leaves and Stage 3 = seedlings beginning to branch.

Statistical analysis

The number of germinated, rotten and empty seeds were checked against the total number of seeds. Where there were discrepancies, seeds were assumed to have germinated, or to be empty seed coats of germinated seeds, depending on whether the cumulative values were more or less than the total number of seeds. Because of this adjustment I report an error rate of 1.5% for *W.whytei* and 1.4% for *W.cedarbergensis* for the counts of germinations, empty and rotten seed. In calculating the final survival rate for the seedlings 15.2% of *W. cedarbergensis* seedlings (190 individuals) were found to be missing and were assumed to have died without being detected, presumably at an early stage or without a recorded death date. The dates of these deaths are unknown and therefore they are not included in monthly deaths but are included when calculating the final percentage of surviving seedlings. Seedling survival was not tested for *W. whytei*.

I used regressions in Excel MS 2007 to determine the relationship between seed viability (%), as the dependent variable and soil nutrients, altitude and distance to nearest neighbour *as the independent variables*. I also used regressions to test the relationship between percentage empty seed and distance to nearest neighbour. The differences between sites in soil nutrients and seed viability were tested with ANOVAs performed in Statistica 13 (Dell Inc. 1984-2015), except for where the data was heteroscedastic, in which case I used Welch's ANOVAs on IBM SPSS Statistics version 23 (IBM corporation, 2015). Welch's ANOVA uses a Games-Howell post hoc test.

I used regressions in Excel MS 2007 to determine the relationship between seed viability (%) and seedling survival (%). Significant differences between seedling deaths at the different seedling stages, as a proportion of total deaths, was determined using a binary logistic generalized linear model on IBM SPSS Statistics version 23 (IBM corporation, 2015). The

Welbedacht population had no deaths for Stage 3, for the model to run, one death was added to one tree of this population for this stage. This is to solve the problem of undefined logits for a population with no variance and is described further in Steenhuisen and Johnson (2012) and Zuur *et al* (2009). The estimated true population means were back-transformed using SigmaPlot version 8.0 (2002 SPSS Inc.) and then graphed in MS Excel 2007.

Results

Widdringtonia whytei

Seed viability

Widdringtonia whytei seed germinated for 70 days after which there was no further germination for a further two weeks when the experiment was ended. Seed viability, as a percentage of the total number of seeds, is between 11.1 and 22.6%. There is an overall significant difference in seed viability between the different populations ($p = 0.046$) for the intial ANOVA (Welch), but the post hoc test (Games-Howell for a Welsh ANOVA) showed no significant difference between pairs of populations. This is probably due to the Madzeka population having a much lower mean viability than the other sites (Figure 2.1). Seed viability across all populations is extremely low and the majority (78.7%) of seed were empty. 3.2% of seeds were full but non-viable (either mouldy or fresh) and only, 18.1%, were full, viable seed (Figure 2.2). The Madzeka site had the highest proportion of empty seed at 84.4% and the lowest proportion of viable seed at 11.1%. The Sombani site has the lowest proportion, 2.1%, of full, non-viable seed and the Lichenya –Kangazani site has the highest percentage of viable seed at 22.6% (Figure 2.2).

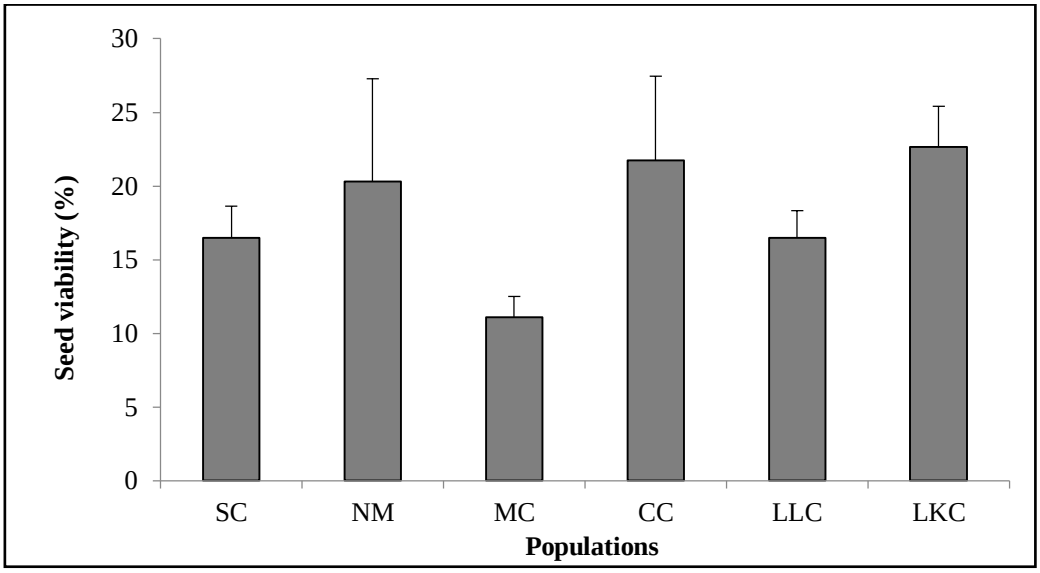

Figure 2.1. The percentage of viable seed of *W. whytei* for each site SC –Sombani, NM- Nomoso Pools, MC- Madzeka, CC – Chinzama, LLC – Lichenya Limbe, LKC – Lichenya Kangazani. There are no significant differences between sites.

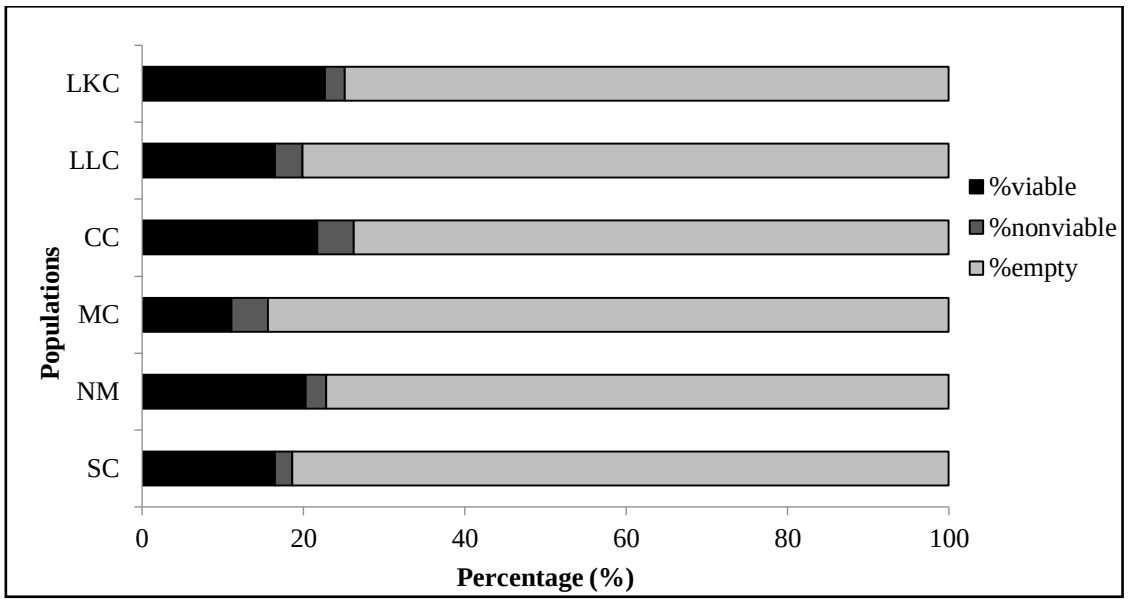

Figure 2.2. The percentage of full but non-viable seed, empty seed and viable seed across populations of *W. whytei* on Mount Mulanje. SC –Sombani, NM- Nomoso Pools, MC Madzeka, CC – Chinzama, LLC – Lichenya Limbe, LKC – Lichenya Kangazani.

The average percentage of viable seed per population is not significantly correlated with distance to nearest neighbour ($R^2 = 0.41$; $p = 0.17$) even though distance to nearest neighbour is significantly different between populations ($p = 0.007$, Fig. 2.3). There is also no significant correlation between empty seed and distance to nearest neighbour. The average distance to nearest neighbour is between 2.5 m (Chinzama) and 11.4 m (Madzeka**)**. The data are highly variable, particularly for the Chinzama and Nomoso Pools sites (Fig. 2.1).

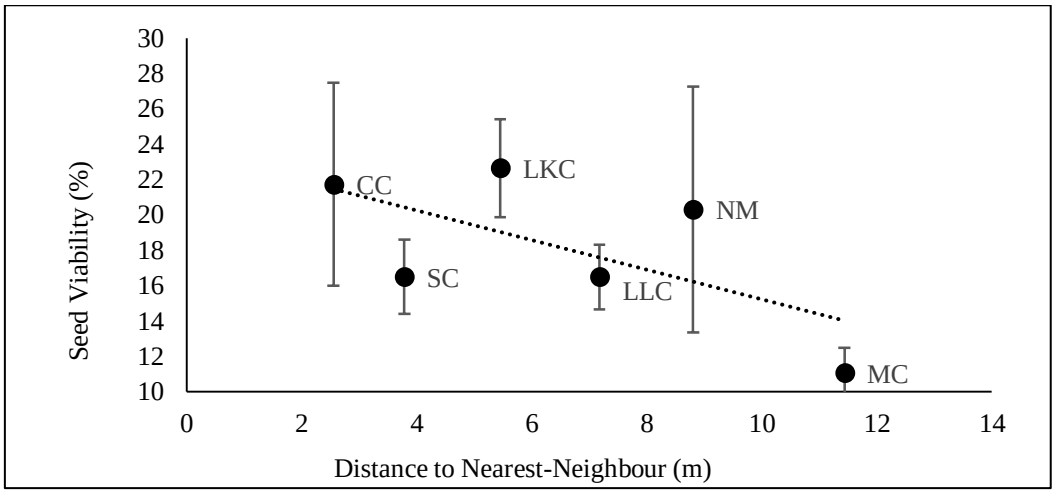

Figure 2.3 Mean and standard error for seed viability, relative to the distance to nearest neighbour, ($R^2 = 0.4089$, $p = 0.172$), for populations of *W. whytei* on Mount Mulanje. SC –Sombani, NM- Nomoso Pools, MC- Madzeka, CC – Chinzama, LLC – Lichenya - Limbe, LKC – Lichenya – Kangazani.

Soil nutrients

The Lichenya- Kangazani site has the highest percent soil nitrogen (2.29%) and carbon (37.69%), while the Nomoso Pools site has the highest proportion of phosphorous (133.66 mg/kg) but the lowest percentage of carbon (23. 82%). The Chinzama site has the lowest levels of nitrogen (1.47%) and phosphorous (73%). The percentage of soil nitrogen, carbon and phosphorous is not significantly different between sites and these nutrients are also not significantly correlated with seed viability.

Table 1: Means and standard error (in brackets) for soil nutrients on Mount Mulanje.

	Nitrogen (%)	**Phosphorous (mg/kg)**	**Carbon (%)**
Sombani	**2.07** (0.10)	**90.66** (8.45)	**36.14** (6.07)
Nomoso Pools	**1.8** (0.26)	**133.66** (44.13)	**23.82** (3.84)
Chinzama	**1.47** (0.56)	**73** (10.97)	**25.28** (10.41)
Madzeka	**2.07** (0.29)	**112.33** (7.51)	**27.73** (3.11)
Lichenya - Limbe	**1.69** (0.44)	**108.33** (1.45)	**31.09** (9.74)
Lichenya - Kangazani	**2.29** (0.18)	**78.33** (44.13)	**37.69** (1.75)

Altitude

The site with lowest altitude is Lichenya-Limbe at 1885 m and Chinzama is the highest altitude site at 2148 m. This gives a range of 263 m with an average of 53 m elevation increase between sites. The altitudinal range for the populations of *W. whytei* is roughly half that measured by Burrows and Allen (1991) and Platt for another southern conifer, *Northofagus menziesii*, and I estimated this was a sufficient range to detect any changes in seed viability with altitude. I found, however, no significant correlation between seed viability and altitude for *W. whytei* ($R^2 = 0.309$, $p = 0.241$).

Widdringtonia cedarbergensis

Seed viability

Germination of *W. cedarbergensis* seeds continued for 128 days after which I ended the experiment, as germination rates had slowed. The average seed viability for all individuals is 44% with the Sneeuberg site having the highest seed viability (62.5%) and Heuningvlei - mountains the lowest (28.7%). The Sneeuberg site is significantly different from the Heuningvlei-mountains ($p < 0.05$) site, but not significantly different from the Welbedacht, De Rif and Heuningvlei-vlakte sites. The Welbedacht, De Rif and Heuningvlei- vlakte sites

are, however, not significantly different from the Welbedacht and Heuningvlei- mountains sites (Fig. 3.1). Empty seed accounts for, on average, 50.2% of the total number of seeds from all sites (Fig. 3.2). The Sneeuberg population also has the lowest proportion of filled, non-viable seed (6.2%) and the lowest proportion of empty seed (31.3%). Heuningvlei-mountains has the highest proportions of full, non-viable (13.9%) and empty seed (57.4%) (Fig. 3.2). Although, the average distance to nearest-neighbour is significantly different ($p = 0.001$) between sites, there is no correlation between seed viability and distance to nearest neighbour. There is also no relationship between empty seed and distance to nearest neighbour.

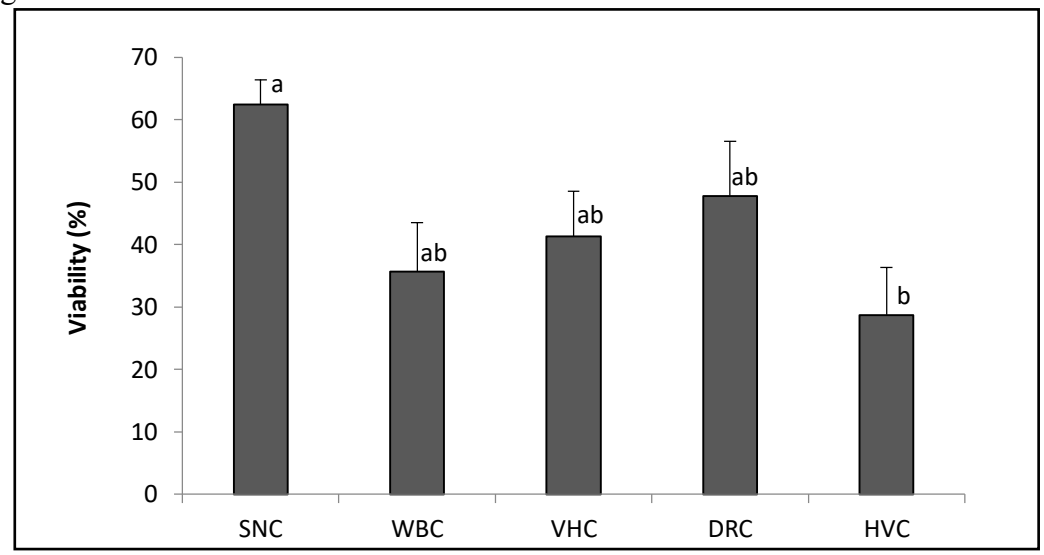

Figure 3.1 Means and standard error for seed viability across populations of *W. cedarbergensis*, with empty seed included. SNC – Sneeuberg, WBC – Welbedacht, VHC- Heuningvlei (vlakte), DRC – De Rif and HVC- Heuningvlei (mountains). Population means which have the same letters are not significantly different from each other ($p = 0.05$).

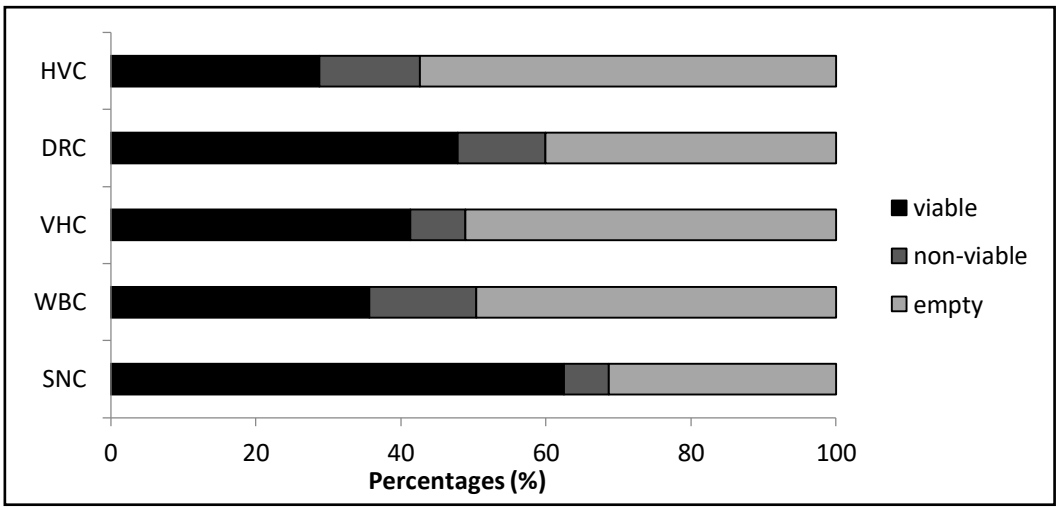

Figure 3.2 The percentage of full but non-viable seed, empty seed and viable seed across populations of W. cedarbergensis. SNC – Sneeuberg, WBC – Welbedacht, VHC- Heuningvlei (vlakte), DRC – De Rif and HVC- Heuningvlei (mountains).

Soil nutrients

Soil collected away from trees is not significantly different between sites in percentage carbon but it is significantly different when collected from under trees ($p < 0.05$). There was a significant difference between sites in percentage phosphorous in soil collected away from trees ($p < 0.05$) but no significant difference in soil from under trees (Table 2). The percentage nitrogen in soil collected away from ($p < 0.05$) and under ($p < 0.05$) trees was significantly different between sites with the Sneeuberg site having the highest percentage of soil nitrogen (0.39%) under trees and the highest seed viability (62%) (Table 2). Despite this, seed viability is not significantly correlated with soil nitrogen or carbon from under trees or soil phosphorous or nitrogen away from trees.

Table 2: Total soil nutrients for samples taken under and away from trees. Standard errors are in brackets.

	Under			Away		
Sites	**Nitrogen (%)**	**Phosphorous (mg/kg)**	**Carbon (%)**	**Nitrogen (%)**	**Phosphorous (mg/kg)**	**Carbon (%)**
Sneeuberg	**0.39** (0.11)	**30** (7.21)	**10.64** (3.22)	**0.11** (0.05)	**10** (1.53)	**2.95** (1.41)
Welbedacht	**0.33** (0.05)	**60.33** (29.24)	**6.91** (1.51)	**0.15** (0.04)	**59** (48.00)	**3.2** (0.72)
Heuningvlei -vlakte	**0.06** (0.01)	**14.33** (5.36)	**1.39** (0.30)	**0.01** (0.00)	**3.67** (0.67)	**0.3** (0.11)
De Rif	**0.03** (0.00)	**13.33** (3.71)	**0.66** (0.05)	**0.05** (0.01)	**15** (1.00)	**1.01** (0.15)
Heuiningvlei- mountains	**0.27** (0.08)	**14** (4)	**6.86** (3.05)	**0.13** (0.03)	**15.67** (10.73)	**3.27** (0.99)

Altitude

Heuningvlei–vlakte is the site with lowest altitude of 952 m while Sneeuberg is the highest at 1351 m. This gives a range of 399 m with an average of 100 m between the five sites. The 400 m range in elevation for *W. cedarbergensis* is similar to that for *Northofagus menziesii* (Burrows and Allen 1991) and I estimated that this would be sufficient to detect any changes in seed viability with altitude. Even though the Sneeuberg site has both the highest seed viability (63%) and the highest altitude (1351 m) seed viability is not significantly correlated with altitude, with only a weak, non-significant increase in seed viability with an increase in altitude.

Seedling survival

The majority of seedlings died in the first life stage (germination to emergence of cotyledons) for all populations (Figure 3.3). The percentage of deaths decrease at Stage 2 (secondary leaves), with the lowest proportion of deaths occurring at Stage 3 (first branches form). The proportion of deaths is significantly higher in Stage 1 than in Stage 2 for all populations. Proportion of deaths at Stage 3 is consistently less than Stage 2, but this is only significantly

different for Heuningvlei -vlakte. De Rif and Sneeuberg have the highest proportions of deaths in Stage 3. The true means are 7.5% and 10% respectively, but this is not significantly different from Stage 3 in other populations (Figure 3.3). There is no correlation between the percentage of surviving seedlings and seed viability for *W. cedarbergensis*.

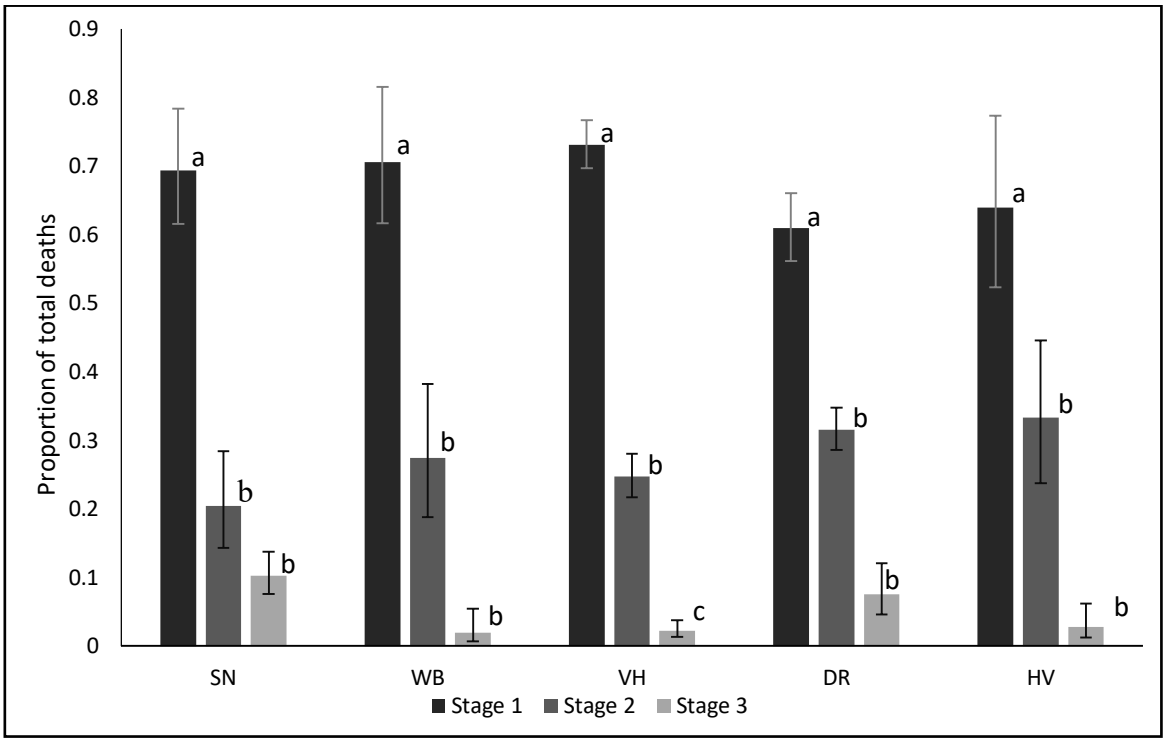

Figure 3.3 Proportion of deaths at the different life stages of *W. cedarbergensis* seedlings, across populations. Values are the true population means and standard errors calculated for the populations using a binary logistic GLM. Means for the different stages which have the same letters are not significantly different from each other (p = 0.05). Letters indicate significant differences between stages ***within*** a site. The same stage across sites are not significantly different.

Discussion

Both *Widdringtonia whytei* and *W. cedarbergensis* are critically endangered and it is likely that as the populations of these two species decrease, distance between adult neighbours will increase. As both species show evidence for poor regeneration, I hypothesized that low regeneration is the result of a decrease in pollination events and seed viability, as a consequence of increased distances to nearest neighbour. To test this hypothesis, I determined if populations with greater distances to nearest neighbour (i.e. lower density) also have reduced seed viability. For *W. whytei* on Mt Mulanje, distance to nearest neighbour is significantly different between sites but not for *W. cedarbergensis* in the Cedarberg mountains. There is, however, no significant correlation between seed viability and distance to nearest neighbour for either *W. whytei* or *W. cedarbergensis*. There was also no significant correlation between the proportion of empty seeds and distance to nearest neighbour for

either species. This does not support the hypothesis that these two *Widdringtonia* species are pollen-limited. My results suggest that pollen dispersal may not be a limitation on seed viability. Pollen limitation is sometimes only detected in very low density populations (Steven and Waller 2007). In addition, pollen limitation can cause a reduction in the number of pollen contributors, but not necessarily in pollination events (Sork *et al* 2002). It is also important to note that the large variation in seed viability for *W. whytei* may be obscuring any relationship between seed viability and distance to nearest neighbour. Seed viability is known to be highly variable in *W. whytei* (Chanyenga *et al* 2011b). This high variability could be a result of micro-site conditions associated with individual trees. For example, there is some evidence for seed viability being limited by drought (García *et al* 2000) and if individual trees have lower water-availability this could lead to high variability in seed viability within a population.

Although, my two study species may be different in terms of population density they are both likely to experience the effects of global change. Anthropogenic climate change and associated temperature increases are considered to increase drought and physiological stress, reduce seedling survival, and increase detrimental wildfires and pest-related mortality in conifers and other trees (Allen 2010; García *et al* 2000; Guarín and Taylor; Souza *et al* 2010). Generally, temperature decreases and rainfall increases with an increase in altitude (Barry and Chorley 2010). *W. whytei* has a 263 m range between the highest and lowest site examined, while *W. cedarbergensis* has a 399 m range which, assuming a decrease in temperature by 6.5°C for every 1000 m increase in altitude (Barry and Chorley 2010), would mean 1.7 °C and 2.6 °C difference for *W. whytei* and *W. cedarbergensis*, respectively. Despite these differences in temperature seed viability was not significantly correlated to altitude for either species. This could be the result of the small differences in altitude (200-400 m) between sites that may not be large enough to detect a change in seed viability.

Several studies have shown the potential for anthropogenically induced nitrogen deposition to either have negative or positive effects, depending on the study site, on growth rates in coniferous forests (Cannell *et al* 1998; Takemoto *et al* 2001). My results however show no difference in percentages of soil nitrogen, phosphorus and carbon between sites for *W. whytei*, while for *W. cedarbergensis* sites were significantly different in terms of percent phosphorous in soil collected away from trees and nitrogen collected under trees. Despite these differences, I found that soil nitrogen, carbon and phosphorous has no influence on seed viability for either *W. whytei* or *W. cedarbergensis*.

In addition to seed viability being affected by altitude, nutrients and distance to nearest-neighbour, I also hypothesized that a decrease in pollination events will mean a decrease in outcross pollen which will result in increased levels of self-pollination. To test this hypothesis, I determined if seed viability is correlated to seedling survival for *W. cedarbergensis*. Although seedling survival was not tested for *W. whytei* it is important to note that within little over a year of germination, all the seedlings had perished from unknown causes.

Of the *W. cedarbergensis* seeds which germinated, 43% survived the first six months. Although the experimental conditions and time-frame may differ from this study, *Picea jezoensis* var *hondoensis* has a survival rate of 20-40%*, Picea glauca* 20-80%, *Tsuga diversifolia* 40-70%, *Abies balsamea* 20%, (Mori *et al* 2004; Cornett *et al* 1998; Johnstone and Chapin 2006). While it does not appear that this survival rate is uncommon among conifers, several species may achieve significantly higher survival rates than my results show for *W. cedarbergensis*. It is not clear if this 43% survival is characteristic of *W. cedarbergensis* or if this is a result of lower population densities causing a pollen limitation, and resulting in self-pollination and weaker offspring.

Seedling survival in *W. cedabergensis* was not significantly correlated to seed viability, which does not support the hypothesis that *W. cedarbergensis* seedlings have higher mortality rates because they are less fit as a result of self-pollination, unless a drop in seed viability is masked by the effect of self-pollination.

Self –pollination does not always cause a failure in seed development (Robledo-Arnuncio *et al* 2004). Depending on the self-compatibility of the species, self-pollination may occur in the absence of cross-pollination (Rajora *et al* 2002). Although there is a reduction in available pollen, specifically outcross pollen, ovules are still pollinated and develop into viable seed. Some self-pollinated ovules may develop and survive to seedling stage, but the fitness of these progeny can be severely reduced (Mosseler *et al* 2000). I hypothesize that low seed viability due to selfing may not always be as easily detected in *W. cedarbergensis* due to mixed pollination (seeds resulting from both self-pollen and cross-pollination). Increases in self-pollination due to reduced population densities may not cause a decrease in seed viability, but rather an increase in the proportion of self-pollinated seeds. It therefore becomes necessary to test levels of self-pollination directly.

Conclusion

In conclusion, my results did not support the hypothesis that seed viability in either *W. whytei* or *W. cedarbergensis* is affected by soil nutrients or altitude. Both species showed high levels of empty seed. Neither *W. whytei* nor *W. cedarbergensis* showed a significant correlation between seed viability and average distance to nearest-neighbour, suggesting that seed viability is not density-dependent at current densities. This does not support the hypothesis of declining populations suffering from additional loss in reproductive ability. *W. cedarbergensis* may, however, be masking the effect of self-pollination, due to trees not being purely selfed or purely outcrossed. Chapter 3 explores the genetic components of the seedlings of *W. cedarbergensis*, using Inter-Simple Sequence Repeats (ISSRs), to assess whether there is any evidence of self-pollination in seedlings.

Chapter 3

Self-Pollination in *W. cedarbergensis*

Introduction

My results for Chapter 2 show that there is 44% seed viability for *Widdringtonia cedarbergensis*, with 43% of seedlings likely to survive the first 6 months after germination. There was, however, no significant correlation between seed viability and seedling survival, giving no evidence that low seed viability was a result of low levels of outcross pollen and high self-pollination rates. This may be because not all viable seed is outcrossed, obscuring the relationship between the two variables. In addition, the high proportion of empty seed are potentially due to pollen limitation, and/or self-pollination. If so, then the decline of *W. cedarbergensis* would be accelerated through pollen limitation and self-pollination. Here, I determine the extent to which *W. cedarbergensis* is self-pollinating, and whether seed viability and seedling survival are affected by self-pollination.

All conifers are wind –pollinated and most are monecious, bearing cones of both sexes on a single tree (Proctor *et al* 1996). The presence of both male and female gametes on the same tree means that self-pollination is possible. Self-pollination is the sexual fertilization of an individual plant's ovules with its own pollen, while cross-pollination is the reception of pollen from another individual (Lowe *et al* 2005; Judd *et al* 2010). Wind-pollination is sometimes limited by the distance between adult trees, as pollen has to travel further and is less likely to reach an individual which is some distance away (Knapp *et al* 2001; Sork *et al* 2002). Increased population fragmentation can also lead to increased self-pollination (Farris and Mitton 1984; Arista and Talvera 1996). This is because fewer pollen donors means that the likelihood of pollen in the pollen cloud being outcross pollen is decreased, and the proportion of self-pollination is increased (Dudash and Fenster 2000; Mimura and Aitken 2007). This is evident in conifers such as *Pinus strobus* that show a strong correlation between stand density and outcrossing rates (Rajora *et al* 2002) and *Fagus sylvatica,* in Sweden, that has higher self-pollination rates in smaller stands (Nilsson and Wästljung 1987).

Self-pollination can be detrimental to the reproduction of a species and the fitness of the resulting offspring (Mosseler *et al* 2000; Husband and Schemske 1994). Loss of fitness due to self-pollination (or other forms of inbreeding) is termed inbreeding depression, which can be evident as a loss of seed viability, increase in empty seeds, and a decrease in offspring

performance (Charlesworth and Charlesworth 1987; Rajora *et al* 2000; O'Connell and Ritland 2005; Robledo-Arnuncio *et al* 2004; Mimura and Aitken 2007; Mosseler *et al* 2000). There are two main theoretical causes of inbreeding depression: overdominance and partial-dominance (Charlesworth and Charlesworth 1987). Both concepts deal with homozygosity and heterozygosity. Heterozygosity is when an individual has two different forms of the same gene (alleles), for a given locus, while homozygosity occurs in individuals with two of the same allele (Lowe *et al* 2005). Overdominance is when heterozygous individuals have greater fitness than their homozygous siblings. The partial-dominance hypothesis predicts an increase in the proportion of deleterious recessive alleles with an increase in inbreeding. These recessive alleles may reduce the fitness of an individual or be fatal (Charlesworth and Charlesworth 1987). Although some plants may show increased fitness with self-pollination, as a result of the increased levels of homozygosity purging semi-lethal or lethal genes from the population (Husband and Schemske 1994), several conifers show a decline in fitness with increased inbreeding. *Pinus patula,* growing as an exotic in Zimbabwe, shows decreased embryo viability when inbred via a combination of self-pollination and close-relative inbreeding (Williams *et al* 1999). In *Pinus strobus*, in Canada, multilocus outcrossing rates are strongly correlated with filled seed production (Rajora *et al* 2002). The proportion of heterozygous loci is positively correlated with trunk growth and cone production in *Pinus attenuata*, and heterozygosity is lower for self-pollinated individuals (Strauss 1986).

Although conifers are considered to be a predominantly outcrossing group, they are capable of self-pollination and some species have mixed mating systems (a combination of outcrossing and self-pollination) or high levels of self-pollination (Williams 2009). Despite the associated inbreeding depression, self-pollinated individuals can produce viable seed and seedlings (Rajora *et al* 2000; Mosseler *et al* 2000; O'Connell and Ritland 2005). *Thuja plicata* has between 5-60% seed viability when artificially pollinated with high proportions of self-pollen (O'Connell and Ritland 2005). *Picea rubens* has estimated values of up to 46% self-pollinated seed in some populations (Rajora *et al* 2000). The proportion of empty self-pollinated seeds of this species is negatively correlated to seedling height, suggesting that self-pollinated individuals survive to the seedling stage (Mosseler *et al* 2000).

Thomas and Bond (1997) suggest that self-pollination with a decline in tree densities and subsequent inbreeding is affecting genetic variation in *W. cedarbergensis*. Their results agree with my findings in Chapter 2, demonstrating high proportions of empty seed, which can be a result of self-pollination and a subsequent inbreeding depression in some populations of

conifers. I did not, however, find any significant correlation between distance to nearest neighbour and seed viability for *W. cedarbergensis*. If *W. cedarbergensis* is self-pollinating and producing viable seed, increased self-pollination rates may not result in reduced seed viability but rather an increase in the number of self-pollinated offspring.

Self-pollination can be assessed through an analysis of genetic variation using inter-simple sequence repeats (ISSRs). These anonymous, dominant markers have been shown to be comparable with AFLPs (amplified fragment length polymorphism) in both information content and reproducibility (e.g. Zietkiewicz *et al* 1994; Fernández *et al* 2002; Arcade *et al* 2000). The low cost and high reproducibility of ISSR's make this a particularly useful method for determining self-pollination (Fang and Roose 1997; Fang *et al* 1997; Prevost and Wilkinson 1999; Goulão and Oliviera 2001; Culley and Wolfe 2001; Goulão *et al* 2001; Bornet and Branchard 2001; Arnau *et al* 2002; Lu *et al* 2006; Słomka *et al* 2011). ISSRs amplify regions between microsatellites, but don't require any previous sequencing information, are often highly polymorphic, and very informative on a per primer basis, making them quicker and cheaper to use than other methods (Zietkiewicz *et al* 1994; Goulão and Oliveira 2001; Fernåndez *et al* 2002; Bentley *et al* 2015; Hao *et al* 2006). ISSRs have been shown to distinguish between rare populations of the succulent *Faucaria tigrina* (Bentley *et al* 2015) and have also been successfully used in diversity studies in several species of conifers, such as *Taxus wallichiana* var. *mairei* and *Cupressus chengiana* in China (Arcade *et al* 2000; Zhang *et al* 2005; Hao *et al* 2006). ISSRs have also been shown to successfully estimate self-pollination and outcrossing rates in *Agicerus corniculatum* (Ge and Sun 1999).

Here, I investigate the extent to which *W. cedarbergensis* is self-pollinating using ISSR markers. I also investigate if seedlings which did not survive the first 6 months after germination are more likely to be self-pollinated, and hence if self-pollination is related to a higher mortality rate. I hypothesize that there will be low genetic variation in *W. cedarbergensis* because of low population densities and that there will be large genetic variation between populations as a result of poor pollen flow.

Methods

Study Site

I examine self-pollination in two of the five sites at which I collected seed in the Cederberg, Sneeuberg (S32°29'25.9'' E19°10'23.1'') and Welbedacht (S32°40'83.3'' E19°17'90.9''). These sites were selected because both are naturally occurring populations of *W. cedarbergensis* while the De Rif and Heuingvlei-vlakte sites are plantations, and the Heuningvlei-mountains site is a mix of natural and planted trees. Both the Sneeuberg and the Welbedacht sites had the lowest population densities and yet are significantly different in terms of seed viability, with Sneeuberg having the highest seed viability (63%) of all the sites.

Collection

Leaves and seeds were collected from each of 6 trees at Sneeuberg and 5 trees at Welbedacht. All leaf material was placed directly into silica gel to preserve the DNA while cones were collected into paper bags. In the laboratory seeds were germinated on agar in a temperature-controlled phytotron with night/day temperatures of 19/20 ± 2 º C and 12 hrs of dark and light. After germination the seedlings were transferred to potting soil (SuperGrow Potting Soil, Stanler Farms) and grown under ambient greenhouse conditions as described in Chapter 2. Leaves were collected from germinated seedlings and dried on silica gel. These seedlings are thus known offspring of the trees from which leaf material and seed were collected in the field. Where possible, 10 seedlings were sampled per tree, 5 that had died in the first 6 months, and 5 that were still alive after 6 months. Seedlings were harvested when it was certain that they would die, and not excluded from the seedling survival counts (Chapter 2).

Extraction and DNA analysis

Dried leaf material was placed in an Eppendorf tube and finely ground using a Retsch MM400 ball bearing mill (Retsch, Haan, Germany). DNA extractions were performed using the CTAB (Cetyltrimethylammonium Bromide) method (Doyle and Doyle 1987 as modified by Gawel and Jarret 1991). The CTAB buffer solution had polyvinylpyrrolidone-40 (PVP-40) added to allow for the high resin content of cedars. PVP is used to remove polyphenols to aid in purer DNA extraction (Friar 2005). The DNA pellets were dissolved in 100 µl of water and further diluted to a 1:10 ratio before amplification of ISSRs in a PCR (Polymerase chain reaction).

I used three UBC (University of British Columbia) ISSR (inter-simple sequence repeats) primers for the PCR. These three primers were chosen for their polymorphism; several primers were run with a small subset of samples and those which showed any band variation amongst this subset of individuals were used for the analysis, but individual bands which were invariant were not excluded. These primers were UBC 812 (GAG AGA GAG AGA GAG AA), UBC 864 (ATG ATG ATG ATG ATG ATG) and UBC 884 (HBH AGA GAG AGA GAG AG). UBC 812 was fluorescently tagged with FAM dye while UBC 864 and 884 were tagged with Hex dye from the department of Molecular Cell Biology, University of Cape Town, South Africa. UBC 812 and UBC 864 were multiplexed and the Kapa Biosystems reagent proportions for PCR were as follows (volumes shown for a single reaction with final reaction concentrations in brackets): H_2O =17.8 µl, Buffer = 3 µl (1X), $MgCl_2$ = 3 µl (2.5 mM), dNTPs = 1 µl (0.33 mM), Primer 1(812) = 1 µl (0.33 µM), Primer 2(864) = 1 µl (0.33 µM) and Taq = 0.2 µl (1 unit). This yielded 27 µl of mastermix which was combined with 3 µl of DNA for the PCR. The following proportions (amounts shown for a single sample) were used for the UBC 884 primer; H_2O = 14.05 µl, Buffer = 2 µl (1X), $MgCl_2$=1.2 µl (1.5 mM), dNTPs = 0.4 µl (0.2 mM), Primer (884) = 0.2 µl (0.1µM) and Taq = 0.15 µl (0.75 units). This yielded 18 µl of master mix which were combined with 2 µl of DNA. The PCRs were run on Applied Biosytems Veriti 96 well and Applied Biosystems 2720 thermal cyclers, using the following temperature profile adapted from Adams *et al* (2003); 94 °C (1:30min) followed by 40 cycles of [91 °C (1min), Anealing temperature 50 °C (2min), 72 °C (2min)] ending off with 72 °C (2min). The PCR product was then run on sequencing gels at the Central Analytical Facility at the University of Stellenbosch, South Africa. Electrophoresis was conducted in an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, California, U.S.A.). Samples were denatured at 95º C for 2 minutes before being transferred to ice for 5 minutes.

Band scoring

The electropherograms were visualised on the computer program Peak Scanner 2 (Applied Biosystems, 2012) and scored manually (Fang and Roose 1997; Joshi *et al* 2000; Culley and Wolfe 2001; Korbin *et al* 2002). Manual scoring was used as bands may be visible below a given threshold as was found when initially an intensity threshold of 50 relative fluorescent units. Some peaks were still clearly visible below this threshold and needed to be included as present. Allowance was also made for samples in which the entire run was weaker than desirable, but where bands could still be determined. Fragments between 15 bp and 740 bp were used, as larger fragments were often indistinct. Sometimes strong bands can appear as

double bands but these were noted and scored as a single band. Bands were scored as present or absent. To give an estimate of repeatability the PCR was repeated for 5 samples. The objective of using five PCR repeats was to determine which bands were reliable and reproducible throughout the data set. Each electropherogram was visualised once, but the samples for which the PCR had been repeated, had two electropherograms which were then compared. Bands that were not consistent across repeats were considered unreliable and discarded, but where they fell below the threshold the band was reassessed for all samples. This process of eliminating faint or unreliable bands has been used in numerous studies (Fang and Roose 1997; Joshi *et al* 2000; Culley and Wolfe 2001; Korbin *et al* 2002; Sica *et al* 2005; Roy *et al* 2006; Zhang *et al* 2006; Słomka *et al* 2011; Sánchez-Gómez *et al* 2013). Based on the repeats 151 bands we discarded and this resulted in 237 reproducible loci across the three primers. Although this number is quite large, it is not unprecedented; for example Bentley *et al* (2015) found 572 ISSR loci using two primers.

Data Analysis

The objective of this chapter is to determine the extent to which *W. cedarbergensis* is self-pollinating. I am not investigating paternity per se but rather the likelihood that the ovule of the maternal parent was pollinated by a pollen grain produced by that same tree and is therefore also the paternal parent. I do this using the likelihood method as implemented in the Colony software package (Version 2.0.5.9, Jinliang Wang, Zoological Society of London, 2008). There are no previous genetic studies on *W. cedarbergensis* using ISSRs, which could give an indication of allelic dropout rates and other genotyping errors. Allelic dropout is the loss of information on the heterozygosity or homozygosity of an individual at a given locus, due to a lack of correct amplification at this locus during the PCR (Wang *et al* 2004). Other errors may occur during amplification, such as the production of false alleles, and during the band scoring and data collation. All of these errors may be accounted for when running the Colony analysis software (Wang *et al* 2004). Due to the lack of previous data on allelic dropout rate this was set at 0 for all markers, but in order to test the robustness of my results, the analyses were rerun with both a 5% and at a 50% error rate estimate for mistyping and other error rates for all markers. This was also because all bands which did not occur in the repeats were removed, theoretically removing much of the potential error. As a result, six maximum likelihood runs were performed for each of the populations, three with the genotyping and mistyping error rate set at 0.05 and three set at 0.5 for all markers. The three runs were automatically merged by the computer program Colony.

All population genetic analyses were performed in Popgene version 1.32 (Yeh *et al* 1997). Only the adults were used for these analyses as seedlings were the corresponding offspring of the adults and therefore not randomly selected from the population. Heterozygosity (H_e) using Nei's (1973) gene diversity, was calculated assuming the populations were in Hardy-Weinberg equilibrium. Total gene diversity, H_T, average gene diversity for populations, H_S, and gene differentiation between populations, Gst, were also measured in Popgene version 1.32 (Yeh *et al* 1997) using Nei (1973). I used a cluster analysis to determine whether individual trees clustered into two genetically distinct populations corresponding to the actual populations from which they were derived. For this, I used a UPGMA (Unweighted Paired Group Method with Arithmetic Mean) which was performed in Popgene version 1.32 (Yeh *et al* 1997). The program uses Nei (1973)'s genetic distance as a distance measure for the UPGMA.

Results

Self-pollination

At a 5% error rate, the Welbedacht site has no seedlings resulting from self-pollination and the Sneeuberg site has two, representing 0% and 5% self-pollination respectively (Table 3.1 and 3.2). When run with a 50% error rate the Welbedacht site has 12% and the Sneeuberg site 8.3% self-pollination. For the higher self-pollination rates, the seedlings showing self-pollination came from only one adult tree in each population.

The Welbedacht population has the lower seed viability (35.7%) of the two populations, almost half that of Sneeuberg which has 62.5% viable seed, but for the runs performed with a 5% error rate, Welbedacht has no seedlings resulting from self-pollinated seed. This does increase with a 50% error rate, but neither population has a high self-pollination rate. When using the 50% error rate, there are 11 self-pollinated seedlings, with 10 out of the 11 dying in the following six month. The majority of seedlings (78%) that died during this period however were not the result of self-pollination.

Table 3.1 Percentage self-pollinated individuals from each tree in the Sneeuberg population 0.05 and 0.5 error rates. N is the number of individuals genotyped.

		0.05 (error rate)		0.5 (error rate)	
Tree Nº.	**N**	**selfed individuals**	**(%)**	**selfed individuals**	**(%)**
SNC1	10	0	0	5	50
SNC2	10	1	10	0	0
SNC3	8	0	0	0	0
SNC4	5	1	20	0	0
SNC5	8	0	0	0	0
SNC6	10	0	0	0	0
Average	**8.5**	**0.33**	**5**	**0.83**	**8.33**

Table 3.2 Percentage selfed individuals for each tree and overall Welbedacht population, for the two error rates used.

		0.05 (error rate)		0.5 (error rate)	
Tree N°	**N**	**Selfed individuals**	**(%)**	**Selfed individuals**	**(%)**
WBC1	10	0	0	6	60
WBC2	9	0	0	0	0
WBC3	10	0	0	0	0
WBC4	8	0	0	0	0
WBC5	8	0	0	0	0
Average	**9**	**0**	**0**	**1.2**	**12**

Population genetics

Expected heterozygosity, H_e, is calculated as a value between 0 and 1; the closer it is to 0 the less heterozygosity is present in the species. This also applies to the average population gene diversity, H_s, and total gene diversity, H_T. Total gene diversity, H_T, gives a measure of the average gene diversity for populations and a measure of the gene diversity between populations (Nei 1973). These values for *W. cedarbergensis* were $H_e = 0.1489$, $H_s = 0.1453$, $H_T = 0.1474$ and G_{ST}, the population differentiation between the Sneeuberg and Welbedacht sites was 0.1497. The polymorphism (P) for *W. cedarbergensis* was 44.7%.

The UPGMA of the two *Widdringtonia cedarbergensis* populations (Figure 4.1) shows that the individuals do not group strongly into populations. There is one individual from each population, WB1 and SN4, which does not fit into either of the larger groups, and each group has at least one member from each population.

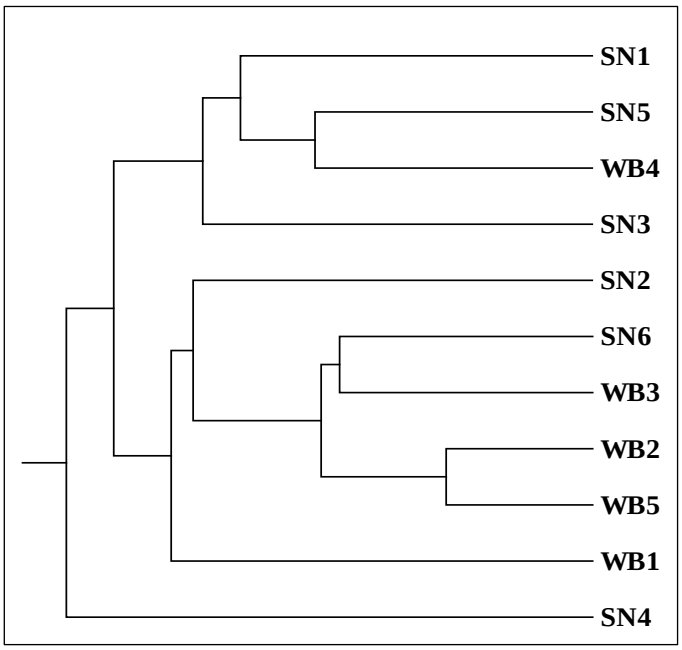

Figure 4.1 A UPGMA showing groupings of individuals.

Discussion

The objective of this chapter was to test the extent of self-pollination in *W. cedarbergensis* and to see if seedlings that did not survive the first 6 months after germination were more likely to be the result of self-pollination. I also determined the population genetics for *W. cedarbergensis* and compare these results with those from several endangered or endemic conifers, to assess the genetic variability within natural populations and to give some indication of gene flow between populations.

Between 5% (low error rate) and 8.3% (high error rate) of seedlings at the Sneeuberg site and 0 to 12% of seedlings at the Welbedacht site have a high probability of being self-pollinated. Whether the high or low error rate is used, these results indicate that *W. cedarbergensis* is not self-pollinating significantly in either population. Rates of self-pollination increase only slightly when the 50% error rate is used indicating that these results are robust. Due to the small number of self-pollinated seedlings found it was not possible to assess self-pollination relative to seed viability and seedling survival.

Self- pollination can be highly variable between populations and across individuals (Arista and Talvera 1996). With a total of 11 parent trees from two populations the small sample sizes used in this study may be a limitation in determining self-pollination for *Widdringtonia cedarbergensis*, however, given the consistency of the results across all samples, it is likely

that these are representative of the species. This would suggest that self-pollination in *W. cedarbergensis* is possible, and does occur, but it is not the predominant mating system at current population densities.

Although these results disagree with the suggestion that *W. cedarbergensis* has low genetic variation due to self-pollination (Thomas and Bond 1997), it is consistent with the high outcrossing rates found in several other conifer species. For example, *Pinus sylvestris* has a 90% outcrossing rate (Robledo-Arnuncio et al 2004), *Picea engelmannii* has 93% and *Abies lasiocarpa* has an 89% outcrossing rate (Shea 1987). This study focused on the likelihood of self-pollination which was assessed by determining the probability that both the male and female gamete was derived from the same tree that the maternal paternal was also the paternal parent, so we cannot discount close relative or biparental inbreeding.

In the absence of self-fertilization avoidance mechanisms in many conifers, high observed outcrossing rates are likely due to post-pollination control such as polyembryony (Williams *et al* 2001; Williams 2009). Polyembryony is the division of the ovule into two or more archegonia, which may be fertilized by different pollen donors, introducing competition in the embryonic stages of development (Sorensen 1982). In the event that one is self-pollinated and the other cross-pollinated, the embryo with greater genetic fitness will out-compete the inferior embryo. This means that although there may be high levels of self-pollination at fertilization, few of these selfed embryos survive, effectively resulting in outcrossing (Sorensen 1982). Although it is not known if it plays a role in preventing self-pollination, polyembryony was observed in *Widdringtonia* during the germination experiments, and this may potentially account for low self-pollination rates. Another possibility is that self-pollination results in empty seed. The results of my analysis in Chapter 2 show high levels of empty seed for *W. cedrbergensis*. Empty seed is regarded as the result of self-pollination in some species (Rajora *et al* 2002), however, empty seed was not correlated with distance to nearest neighbour and is therefore not likely to be a result of pollen limitation or self-pollination in my study species.

In order to contextualise my results for population genetics of *W. cedarbergensis*, I compare my population genetics results to those obtained for 11 other species of conifer (Table 4). The criterion for comparison was research investigating endangered and/or patchily distributed conifers using a dominant marker (either AFLPs or ISSRs) and which presented some measure for heterozygosity, genetic distance between populations and polymorphism.

For dominant markers, a band at a single locus is considered polymorphic if it is present in some individuals and absent in others. This is expressed as a percentage of loci which are polymorphic for the species and gives an indication of genetic variation (Yeh *et al* 1999). The average level of polymorphism (P = 44.7%) in *W. cedarbergensis* is much lower than the average (P = 78%, SD = 32) for the review. It matches that of *Juniperus phoenicea* (45%) and the only conifer listed with lower polymorphism is the critically endangered *Pinus squamata* in Yunnan province, China, with only 12% polymorphism. This indicates low genetic diversity in *W. cedarbergensis.* It is, however, important to note that this low polymorphism may also be due to the design of the experiment. As the primary aim was to detect self-pollination, emphasis was placed on genotyping known offspring and their mothers, however, these offspring could not be included in the assessment of population genetics as their relatedness would have greatly biased the results. As a result, 11 adults in total from 2 populations were used and this may underestimate the polymorphism somewhat.

Table 4: Heterozygosity, polymorphism and genetic distance for other conifers compared with *W. cedarbergensis.* Values for polymorphism (P) are rounded off to the nearest percentage. H_e – species level heterozygosity, H_S – average heterozygosity for populations, H_T – total gene diversity, G_{ST} –genetic distance between populations.

Species	**Mark**	**P (%)**	**H_e**	**H_S**	**H_T**	**G_{ST}**		**Reference**
Juniperus phoenicea	ISSRs	45		0.1299	0.148	0.12	Med.	Meloni *et al* 2006
Pseudotaxus chienii	ISSRs	73			0.2118	0.615	China	Su *et al* 2009
Cupressus gigantea	ISSRs	69	0.2		0.19	0.36	Quighai-Tibetan Plateau	Xia *et al* 2008
Cupressus chengiana	ISSRs	99	0.312	0.1631	0.3132	0.479	China	Hao *et al* 2006
Pinus squamata	ISSRs	12	0.029				China	Zhang *et al* 2005
Pinus armandii subsp. xuannhaensis	ISSRs	96	0.302	0.115	0.325	0.647	Vietnam	Tam *et al* 2015
Taxus wallichiana var. *mairei*	ISSRs	98	0.346	0.207		0.406	China	Zhang *et al* 2009
Thuja sutchuenensis	ISSRs	100	0.165	0.15	0.167	0.102	China	Liu *et al* 2013
Tetraclinis articulata	ISSRs	98		0.179	0.258	0.31	Med.	Sánchez-Gómez et al 2013
Abies ziyuanensis	AFLP	84	0.263	0.136		0.482	S. China	Tang *et al* 2008
*Keteleeria davidiana*var. *formosana*	AFLP	84	0.233				Taiwan	Fang *et al* 2013
	Mean	**78**	**0.231**	**0.154**	**0.230**	**0.391**		
	SD	**27.70**	**0.101**	**0.031**	**0.069**	**0.192**		
W. cedarbergensis		**45**	**0.148**	**0.125**	**0.147**	**0.149**	**S. Afr**	**Present study**

The mean expected heterozygosity (H_e) for *W. cedarbergensis*, given by Nei's (1973) genetic diversity, is 0.1489 and is slightly lower than average for the other conifers reviewed (H_e = 0.2313, SD = 0.1015). The only species with lower heterozygosity is the critically endangered *Pinus squamata* (H_e = 0.029) (Zhang *et al* 2005), and it is also very close to that of *Thuja sutchuenensis* (H_e = 0.165) which is also critically endangered with small remnant populations (Liu *et al* 2013). The average gene diversity (H_s) for the two populations of *W. cedarbergensis* (H_s = 0.1253) is similar to the average for the review (H_s = 0.1543) and therefore, is probably quite typical for endangered and/or endemic conifers. The total gene diversity, H_T, is 0.1474 is also slightly lower than the average for the reviewed species. This is also consistent with low genetic variability found in endemic and rare plants in general (Hamrick *et al* 1979; Gitzendanner and Soltis 2000).

The genetic diversity is, however, significantly lower than the 0.39 reported by Thomas and Bond (1997) for *W. cedarbergensis*. The difference may be due to the number of trees sampled, as Thomas and Bond (1997) collected cones from 30-40 trees per site and from several sites. They also sampled seedlings, while the population genetics which I have presented here are derived from adult trees. The life history stage at which samples are collected can be important in influencing measured genetic variation. In *Pinus ponderosa*, seedlings can show higher levels of heterozygosity than seeds, due to the loss of less fit siblings over time (Farris and Mitton 1984). The main difference, however, is likely to be because of the difference in marker systems used. ISSRs were used in this study while Thomas and Bond (1997) used isozymes. Several studies show discrepancies when comparing across different markers (Powell *et al* 1996; Nybom 2004).

The genetic distance between *W. cedarbergensis* (G_{st} = 0.1492) populations is less than half that for the reviewed species (G_{ST} = 0.3913) but similar to the F_{st} – value (the codominant equivalent of G_{ST}) which Thomas and Bond (1997) reported for *W. cedarbergensis* (F_{st} = 0.176). This means that the two *W. cedarbergensis* populations analysed are genetically quite similar to each other, whereas other endangered and/or endemic species tend to be far more differentiated. Isolated populations can become dissimilar over time due to genetic drift, but populations which have high gene flow between them tend to be more similar (Mitton and Williams 2006). Good gene flow between populations occurs through pollen and/or seed dispersal and pollen usually covers greater distances in conifers (Ge *et al* 2005). My results suggest that there is good gene flow, most likely in the form of pollen movement, between populations. An alternate reason why the two *W. cedarbergensis* populations are not strongly

diverged may be that they have only recently been separated through population reduction and not enough time has passed for populations to diverge (Loveless and Hamrick 1984; Thomas and Bond 1997). The mode of chloroplast inheritance in *Widdringtonia* has not been investigated, but it is paternally inherited in the closely related *Callitris* (Sakaguchi *et al* 2014). If this is also the case for *Widdringtonia*, future studies may be able to elucidate if population similarities are due to high pollen-mediated gene flow or remnant historic structuring.

Conclusion

My results suggest that self-pollination can occur in *W. cedarbergensis* but that the current density of mature trees does not result in a high rate of self-pollination. This may be due to polyembryony or an absence of distance-related pollen limitation. This is a positive sign in terms of conservation as it means that self-pollination is not accelerating the decline of the species. However, it does not exclude other forms of inbreeding, such as biparental or close-relative inbreeding, which may still be a concern in these sparse populations. The population genetics reveal relatively low expected heterozygosity but little genetic differentiation between populations, suggesting that the species is not genetically diverse, but that this is constant across populations, and that the populations are as yet not differentiating due to isolation.

Chapter 4

Synthesis

Globally, trees, including many species of conifer, are threatened by increases in wildfires, often associated with anthropogenic climate change (Allen *et al* 2010). In addition many conifers are under threat from unsustainable harvesting (Berg *et al* 1995; Zhang *et al* 2005; Liu *et al* 2013). The two endangered African cedars in my study, *Widdringtonia whytei* and *W. cedarbergensis*, are both experiencing rapid population decline as a result of either global climate change, past or present logging or increased fire frequency (White 2013; Chapman 1995; Makungwa 2004; Manders 1987b). In addition to dramatically reduced populations, both species show evidence of poor recruitment and establishment (Higgins *et al* 2001; Lawrence *et al* 1994; Fox 2003; Makungwa 2004). A decrease in population density can result in a decrease in viable seed production and an increase in the likelihood of self-pollination, due to a lower availability of outcrossed pollen, and self-pollination can result in less fit offspring (Farris and Mitton 1984; Arista and Talvera; Robledo-Arnuncio *et al* 2004). The potential for the decline of a species to be accelerated through reduced reproduction and establishment, is a concern for both *W. whytei* and *W. cedarbergensis.*

Anthropogenic temperature changes may drive species upslope (Haplin 1997; Midgley *et al* 2003; Hannah *et al* 2005) where temperatures are generally cooler and rainfall higher (Barry and Chorley 2010), where conditions may become more amenable for survival while at lower altitudes reproduction may decrease. Seed viability may also increase with increased resource availability, which may in turn be influenced by local changes in nitrogen deposition (Cannel *et al* 1998; Takemoto *et al* 2001; Wallace and O'Dowd 1989; Campbell and Halama 1993). I tested the relationship between seed viability and altitude, as well as between seed viability and soil nitrogen, phosphorous and carbon. These variables did not explain seed viability, suggesting that while resource availability may still affect other aspects of tree health this is not currently affecting seed production and viability.

The negative effect of pollen limitation on seed production has been demonstrated in several conifer species (Allison 1990; Smith *et al* 1990; Mosseler *et al* 2000; Rajora *et al* 2002). To determine if my study species are pollen limited, I examined the effect of increasing distance to nearest –neighbour on seed viability for both *W. cedarbergensis* and *W. whytei*. I also tested if reduced seed viability, was related to self-pollination and consequent decline in seedling survival in *W. cedarbergensis*. My results show that seed viability was not significantly

correlated with average distance to nearest-neighbour for either *W. whytei* or *W. cedarbergensis* nor was it related to seedling survival in *W. cedarbergensis*.

Based on these results, I hypothesised that viable selfed seeds were masking this relationship between seed viability and distance to nearest neighbour, and tested for this using inter simple sequence repeats. This hypothesis is based on research by Thomas and Bond (1997) who suggest that low genetic variation in *W. cedarbergensis* is due to ineffective pollen transfer and resulting self-pollination. The results show that while some self-pollination does occur in *W. cedarbergensis*, it is not common and it is a predominantly outcrossing species which supports the general assertion that conifers are predominantly outcrossing (Williams 2009). Although we cannot rule out close relative or biparental inbreeding with low self-pollination rates, populations were not genetically diverged, suggesting adequate pollen flow. Further research into rates of pollen flow between populations of *W. cedarbergensis*, using chloroplast inheritance, would be needed to confirm this. There is also no evidence to suggest that loss of seedling fitness due to self-pollination in *W. cedarbergensis* causes high seedling mortality. These results support my earlier assertion that low seed viability is not correlated with seedling survival or distance to nearest neighbour.

W. cedarbergensis has slightly lower than the average genetic variation but similar to several endangered conifers, such as *Juniperus phoenicea* and *Thuja sutchuenensis* (Meloni *et al* 2006; Liu *et al* 2013). They are, however, dissimilar to the results of Thomas and Bond (1997), who reported much higher genetic variation for *W. cedarbergensis*. This is likely due to the different life stages analysed in the two studies and the different markers which were used (Farris and Mitton 1984; Powell *et al* 1996; Nybom 2004).

There is no evidence for reproductive decline in either *W. cedarbergensis* or *W. whytei* at current population densities nor is *W. cedarbergensis* experiencing high levels of self-pollination. Several studies show density-dependent pollen limitation in conifers and other wind-pollinated trees (Allison 1990; Mosseler *et al* 2000; Rajora *et al* 2002). For example, seed set in *Taxus canadensis* is significantly positively correlated to pollen production and negatively correlated to distance to nearest neighbour. Contrary to this, there is no support for the argument that decreasing distances between parent trees decreases seed production. Pollen limitation is not always simply expressed as density-dependent. For example, in *Pinus contorta*, although there is a positive correlation between densities of airborne pollen and ovule fertilization, low tree density does not necessarily equate to low fertilization rates

(Smith *et al* 1990). This is because these pines express different growth forms at different densities and may place more reproductive effort on pollen rather than ovule production when they are growing far apart, but this reverses when they are growing close together (Smith *et al* 1990), obscuring the expected relationship of density and pollen availability. This may be more applicable to *W. whytei* than *W. cedarbergensis*, as *W. whytei* often grows in dense stands, but may also be found in smaller clusters (Chapman 1995).

Although *W. whytei* and *W. cedarbergensis* populations are experiencing severe declines, and despite the impacts of global change, there are no effects on reproduction. However, in the context of global anthropogenic threats to conifers, it is imperative that every effort is made to understand how to maintain and boost viable populations in the wild so that these species may persist for future generations. Conservation planting efforts should be at aimed at preserving current population densities, in areas protected from fire (Manders 1986a and 1987b) and particularly at higher altitudes where White (2013) suggests fewer adults die. Planting efforts should also take into account the large volume of seedlings needed to compensate for a 43% survival rate in *W. cedarbergensis* and the low survival rate observed in *W. whytei*. Ideally, seed would be collected across several populations to maintain genetic diversity, however, with very low genetic distance between the two *W.cedarbergensis* sites, where time and financial resources are limited, seed could be collected from populations which have higher seed viability, such as at the Sneeuberg site. Future studies should also determine if self-pollination is also absent in *W. whytei.* These studies should focus on maintaining genetic variation within and between populations of *W. cedarbergensis* and confirming that low population differentiation is a result of adequate pollen flow. The results of this future research should be used to determine planting strategies which encourage the maintenance of genetic variation, in the hope that this will increase the species robustness in the face of global change and ensure the persistence of these two iconic tree species.

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Appendix

Appendix 1: Actual seed counts within the various groups of seed for all trees of *W. whytei* examined. SC – Sombani, NM – Nomoso Pools, MC- Madzeka, LC – Lichenya -Limbe, LK – Lichenya - Kangazani.

Tree ID	Number of Cones	Total Number of Seeds	Germinated	Rotten/ Mouldy	Empty
SC1	10	83	12	1	70
SC2	10	92	10	2	80
SC3	10	130	34	6	90
SC4	10	121	18	2	101
SC5	10	98	15	1	82
SC6	10	98	17	2	79
NM1	10	104	5	0	99
NM2	10	108	14	1	93
NM3	9	95	41	9	45
NM4	10	73	2	0	71
NM5	10	61	12	1	48
NM6	9	65	25	2	38
MC1	10	101	13	7	81
MC2	10	131	16	0	115
MC3	10	90	5	9	76
MC4	10	106	14	3	89
MC6	10	104	12	3	89
CC1	10	124	46	7	71
CC2	10	85	12	3	70
CC3	10	94	20	1	73
CC4	10	93	2	10	81
CC5	10	117	20	7	90
CC6	10	101	39	0	62
LLC1	10	148	27	1	120
LLC2	10	84	10	8	66
LLC3	10	98	13	2	83
LLC4	10	115	23	5	87
LLC5	10	141	18	5	118
LLC6	10	101	23	0	78
LKC1	10	88	21	2	65
LKC2	10	88	16	2	70
LKC3	10	74	21	1	52
LKC4	10	117	27	2	88
LKC5	10	84	10	5	69
LKC6	10	92	28	1	63

Appendix 2: Actual counts within the various groups of seed for all trees of W. cedarbergensis examined. Aborted seed was seed smaller than 0.063 cm^2. SN – Sneeuberg, WB – Welbedacht, VH – Heuningvlei- vlakte, DR- De Rif, HV – Heuningvlei – mountains.

Tree ID	Number of Cones	Total Number of Seeds	Germinated	Full, Ungerminated, Viable	Total Viable	Rotten/ Mouldy	Full, Non-Viable	Empty	
								Aborted seed included	Aborted seed excluded
SNC1	5	63	26	0	26	0	1	36	32
SNC2	6	88	55	2	57	0	3	28	27
SNC3	10	114	70	0	70	8	0	36	35
SNC4	10	113	64	2	66	1	8	38	29
SNC5	10	111	76	0	76	0	8	27	27
SNC6	10	118	77	0	77	2	8	31	21
WBC1	10	131	41	3	44	1	41	45	40
WBC2	10	105	27	1	28	0	3	74	60
WBC3	10	108	28	0	28	0	2	78	44
WBC4	10	100	40	0	40	1	21	38	26
WBC5	11	126	31	0	31	9	1	85	65
VHC1	10	97	33	0	33	3	12	49	49
VHC2	10	117	52	0	52	4	4	57	57
VHC3	10	107	69	0	69	1	1	36	26
VHC4	10	102	24	0	24	6	3	69	68
VHC5	10	117	25	0	25	0	3	89	81
VHC6	10	126	61	0	61	5	6	54	46
DRC1	10	128	86	0	86	2	12	28	26
DRC2	10	100	13	0	13	11	0	76	71
DRC3	10	132	67	1	68	3	11	50	44
DRC4	10	115	32	0	32	16	0	67	53
DRC5	10	101	42	0	42	11	0	48	36
DRC6	10	119	59	0	59	8	1	51	14
HVC2	10	96	6	0	6	7	0	83	67
HVC3	10	124	38	0	38	9	1	76	62
HVC4	8	104	37	0	37	14	0	53	43
HVC5	10	113	26	0	26	15	1	71	68
HVC6	10	114	42	0	42	22	2	48	43

Appendix 3: a tabulated guide of the terms used for various groups of seed and the definitions used.

	Total seeds All seeds including viable, non-viable full seed, rotten/mouldy seed and empty seed. For *W. cedarbergensis* calculations which excluded small aborted seed, it is excluded from both the total and the number of empty seeds.						
	Viable			**Non-Viable**			
				Full Non-Viable		**Empty**	
	Germinated	**Full, Ungerminated, Viable**	**Total Viable**	**Rotten/Mouldy**	**Full, Non-Viable**		
Description	Those seeds which germinated	Those seeds which did not germinate, had fresh embryo and female gametophyte and determined viable with the tetrazolium chloride test.	Germinated seed and ungerminated, fresh seed determined viable by tetrazoilum	Seed which had a mouldy or rotten embryo and/or female gametophyte	Ungerminated seed with fresh embryo and female gametophyte but determined non-viable by	Seed which contained no embryo or female gametophyte, which were only comprised of a woody seed coat. This included small, aborted seed with no hollow space inside the	
						Aborted seed included	Aborted seed excluded

Appendix 4: Population mean for seeds per cone for *W. whytei* with the standard error in brackets

	Total Number of Seeds	Germinated	Rotten/ Mouldy	Empty
Sombani	10.4 (0.7)	1.8 (0.3)	0.2 (0.1)	8.4 (0.4)
Nomoso Pools	8.7 (0.8)	1.8 (0.7)	0.2 (0.2)	6.7 (1.0)
Madzeka	10.6 (0.7)	1.2 (0.2)	0.4 (0.2)	9.0 (0.7)
Chinzama	10.1 (0.6)	3.9 (0.7)	0.0 (0.2)	6.2 (0.4)
Lichenya - Limbe	11.5 (1.0)	1.9 (0.3)	0.4 (0.1)	9.2 (0.9)
Lichenya - Kangazani	9.1 (0.6)	2.1 (0.3)	0.2 (0.1)	6.8 (0.5)

Appendix 5: Population means of per cone seed count for *W. cedarbergensis*. Aborted seed was seed smaller than 0.063 cm^2. Standard errors are in brackets SN – Sneeuberg, WB – Welbedacht, VH – Heuningvlei- vlakte, DR- De Rif, HV – Heuningvlei – mountains.

	Total Number of Seeds	Germinated	Full, Ungerminated, Viable	Total Viable	Rotten/ Mouldy	Full, Non-Viable	Empty	
							Aborted seed included	Aborted seed excluded
Sneeuberg	12.1 (0.5)	7.2 (0.5)	0.1 (0.1)	7.3 (0.6)	0.2 (0.1)	0.0 (0.0)	4.2 (0.7)	3.7 (0.6)
Welbedacht	11.2 (0.5)	3.3 (0.3)	0.1 (0.1)	3.4 (0.3)	0.2 (0.2)	0.1 (0.1)	6.2 (0.9)	4.6 (0.6)
Heuningvlei-Vlakte	11.1 (0.4)	4.4 (0.8)	0.0 (0.0)	4.4 (0.8)	0.3 (0.1)	0.0 (0.0)	5.9 (0.7)	5.5 (0.8)
De Rif	11.6 (0.5)	5.0 (1.1)	0.0 (0.0)	5.0 (1.1)	0.9 (0.2	0.0 (0.0)	5.3 (0.7)	4.1 (0.8)
Heuningvlei - Mountains	11.5 (0.6)	3.2 (0.7)	0.0 (0.0)	3.2 (0.7)	1.4 (0.3)	0.0 (0.0)	6.9 (0.6)	5.9 (0.5)

Appendix 6: Per tree values for *W. whytei.* Seed set is the percentage fertilized seed (Viable and non-viable full seed). SC-Sombani, NM-Nomoso Pools, MC-Madzeka, LC-Lichenya-Limbe, LK-Lichenya-Kangazani.

Tree ID	Nearest-neighbour (m)	Empty (%)	Non-viable, full (%)	Viable (%)	Seed set (%)
SC1	4.56	84.3	1.2	14.5	15.7
SC2	4.53	87.0	2.2	10.9	13.0
SC3	3.07	69.2	4.6	26.2	30.8
SC4	4.65	83.5	1.7	14.9	16.5
SC5	2.13	83.7	1.0	15.3	16.3
SC6	3.75	80.6	2.0	17.3	19.4
NM1	6.81	95.2	0.0	4.8	4.8
NM2	11.2	86.1	0.9	13.0	13.9
NM3	11.2	47.4	9.5	43.2	52.6
NM4	2.81	97.3	0.0	2.7	2.7
NM5	2.81	78.7	1.6	19.7	21.3
NM6	18	58.5	3.1	38.5	41.5
MC1	8.7	80.2	6.9	12.9	19.8
MC2	3.74	87.8	0.0	12.2	12.2
MC3	3.74	84.4	10.0	5.6	15.6
MC4	10	84.0	2.8	13.2	16.0
MC6	31	85.6	2.9	11.5	14.4
CC1	2.4	57.3	5.6	37.1	42.7
CC2	1.76	82.4	3.5	14.1	17.6
CC3	1.76	77.7	1.1	21.3	22.3
CC4	3.74	87.1	10.8	2.2	12.9
CC5	1.2	76.9	6.0	17.1	23.1
CC6	4.46	61.4	0.0	38.6	38.6
LLC1	5.7	81.1	0.7	18.2	18.9
LLC2	11	78.6	9.5	11.9	21.4
LLC3	3.16	84.7	2.0	13.3	15.3
LLC4	9.3	75.7	4.3	20.0	24.3
LLC5	9.25	83.7	3.5	12.8	16.3
LLC6	4.64	77.2	0.0	22.8	22.8
LKC1	4.25	73.9	2.3	23.9	26.1
LKC2	4.1	79.5	2.3	18.2	20.5
LKC3	10.2	70.3	1.4	28.4	29.7
LKC4	5.57	75.2	1.7	23.1	24.8
LKC5	3.78	82.1	6.0	11.9	17.9
LKC6	4.8	68.5	1.1	30.4	31.5

Appendix 7: Per tree values for *W. cedarbergensis.* Seed set is the percentage fertilized seed (Viable and non-viable full seed). Distance to nearest neighbour is averaged for four nearest neighbours. SN – Sneeuberg, WB – Welbedacht, VH- Heuningvlei- vlakte, DR- De Rif, HV – Heuningvlei – mountains.

Tree ID	Nearest-neighbour (m)	Empty (%)	Non-viable, full (%)	Viable (%)	Seed set (%)
SNC1	6.9	57.1	1.59	41.3	42.9
SNC2	4.6	31.8	3.41	64.8	68.2
SNC3	38.0	31.6	7.02	61.4	68.4
SNC4	120.0	33.6	7.96	58.4	66.4
SNC5	37.3	24.3	7.21	68.5	75.7
SNC6	8.5	26.3	8.47	65.3	73.7
WBC1	38.8	34.4	32.06	33.6	65.6
WBC2	41.3	70.5	2.86	26.7	29.5
WBC3	37.5	72.2	1.85	25.9	27.8
WBC4	16.5	38.0	22.00	40.0	62.0
WBC5	109.1	67.5	7.94	24.6	32.5
VHC1	3.2	50.5	15.46	34.0	49.5
VHC2	7.1	48.7	6.84	44.4	51.3
VHC3	4.3	33.6	1.87	64.5	66.4
VHC4	6.2	67.6	8.82	23.5	32.4
VHC5	4.9	76.1	2.56	21.4	23.9
VHC6	9.1	42.9	8.73	48.4	57.1
DRC1	5.7	21.9	10.94	67.2	78.1
DRC2	5.0	76.0	11.00	13.0	24.0
DRC3	6.4	37.9	10.61	51.5	62.1
DRC4	4.1	58.3	13.91	27.8	41.7
DRC5	8.7	47.5	10.89	41.6	52.5
DRC6	4.8	42.9	7.56	49.6	57.1
HVC2	13.7	86.5	7.29	6.3	13.5
HVC3	6.5	61.3	8.06	30.6	38.7
HVC4	13.3	51.0	13.46	35.6	49.0
HVC5	26.4	62.8	14.16	23.0	37.2
HVC6	34.8	42.1	21.05	36.8	57.9

Appendix 8: Average distance to nearest neighbour and standard errors for population of both *Widdringtonia species*. Values for *W. cedarbergensis* are derived from the four nearest neighbours.

W. whytei			***W. cedarbergensis***		
	Average Distance to Nearest Neighbour (m)	**Standard Error**		**Average Distance to Nearest Neighbour (m)**	**Standard Error**
Sombani	3.8	0.4	Sneeuberg	35.9	17.9
Nomoso Pools	8.8	2.4	Welbedacht	48.6	15.8
Madzeka	11.4	5.1	Heuningvlei-Vlakte	5.8	0.9
Chinzama	2.6	0.5	De Rif	5.8	0.7
Lichenya - Limbe	7.2	1.3	Heuningvlei - Mountains	18.9	5.1
Lichenya - Kangazani	5.5	1.0			

Appendix 9: Population mean of percentage seed for *W. whytei*

	Empty (%)	Full, non-viable	Viable (%)
SC	81.4	2.1	16.5
NM	77.2	2.5	20.3
MC	84.4	4.5	11.1
CC	73.8	4.5	21.7
LLC	80.2	3.4	16.5
LKC	74.9	2.4	22.6
Mean	78.6	3.2	18.1

Appendix 10: Population mean of percentage seed for *W. cedarbergensis* (excluding small aborted seed).

	Empty (%)	Full, non-viable (%)	Viable (%)
Sneeuberg	31.3	6.2	62.5
Welbedacht	49.6	14.8	35.6
Heuningvlei-Vlakte	51.1	7.6	41.3
De Rif	40.0	12.2	47.8
Heuningvlei - Mountains	57.4	13.9	28.7
Mean	45.9	10.9	43.2